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(54) Title: DETECTION OF CERVICAL NEOPLASIAS USING FLUORESCENCE SPECTROSCOPY

(57) Abstract

A method for detecting tissue abnormality, particularly precancerous cervical tissue, through fluorescence spectroscopy is disclosed. *In vitro* fluorescence measurements over a variety of different fluorescence spectra are used to screen tissue samples. Using a principal component analysis (PCA), it is possible to discriminate between normal and dysplastic tissues with relatively low false-positive and false-negative results.

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DETECTION OF CERVICAL NEOPLASIAS USING FLUORESCENCE SPECTROSCOPY

BACKGROUND OF THE INVENTION

The field of the invention relates to optical methods used for the screening and diagnosis of tissue abnormalities. In particular, the invention relates to the use of fluorescent spectroscopy to detect cancerous and precancerous tissues of the cervix.

Cervical cancer is the second most common malignancy in women worldwide, exceeded only by breast cancer and in the United States, it is the third most common neoplasm of the female genital tract - 15,000 new cases of invasive cervical cancer and 55,000 cases of carcinoma in situ (CIS) were reported in the U.S. in 1994. In 1994, an estimated 4,600 deaths occurred in the United States alone from cervical cancer. However, in recent years, the incidence of pre-invasive squamous carcinoma of the cervix has risen dramatically, especially among young women. Women under the age of 35 years account for up to 24.5% of patients with invasive cervical cancer, and the incidence is continuing to increase for women in this age group. It has been estimated that the mortality of cervical cancer may rise by 20% in the next decade unless further improvements are made in detection techniques.

The mortality associated with cervical cancer can be reduced if this disease is detected at the early stages of development or at the pre-cancerous state (cervical intraepithelial neoplasia (CIN)). A Pap smear is used to screen for CIN and cervical cancer in the general female population. This technique has a false-negative error rate of 15-40%. An abnormal Pap smear is followed by

colposcopic examination, biopsy and histologic confirmation of the clinical diagnosis. Colposcopy requires extensive training and its accuracy for diagnosis is variable and limited even in expert hands. A diagnostic method that could improve the performance of colposcopy in the hands of less experienced practitioners, eliminate the need for multiple biopsies and allow more effective wide scale diagnosis could potentially reduce the mortality associated with cervical cancer.

Recently, fluorescence, infrared absorption and Raman spectroscopies have been proposed for cancer and precancer diagnosis. Many groups have successfully demonstrated their use in various organ systems. Auto- and dye-induced fluorescence have shown promise in recognizing atherosclerosis and various types of cancers and precancers. Many groups have demonstrated that autofluorescence may be used for differentiation of normal and abnormal tissues in the human breast and lung, bronchus and gastrointestinal tract. Fluorescence spectroscopic techniques have also been investigated for improved detection of cervical dysplasia.

Despite these advances, there remains a need for diagnostic methods with improved accuracy and ease of application that also provide more rapid results. Such methods will permit earlier diagnosis, more effective patient management and, potentially, reduce mortality.

SUMMARY OF THE INVENTION

Thus, it is an objective of the present invention to provide improved methods for the early detection of neoplasia. In particular, it is an objective of the present invention to provide improved spectroscopic methods for the identification of abnormal cervical

tissue, thereby providing a rapid, accurate and simple method for detecting cancerous or precancerous cervical tissue.

In satisfying these and other objectives, there is provided a method for the optical diagnosis of tissue abnormalities. In one embodiment, the present invention provides for the detection of tissue abnormality in a tissue sample *in vitro* by illuminating a tissue sample with a series of electromagnetic radiation wavelengths selected to cause the tissue sample to produce a series of fluorescence intensity spectra indicative of tissue abnormality. The fluorescence intensity spectra emitted from the tissue sample as a result of illumination with the electromagnetic radiation are detected. Then, a probability that the tissue sample is normal or abnormal is calculated from the fluorescence intensity spectra.

The invention further contemplates that the calculations include principal component analysis of the spectra, relative to a plurality of preprocessed spectra obtained from tissue samples of known diagnosis. The invention also contemplates normalizing the spectra, relative to a maximum intensity within the spectra, and mean-scaling the spectra as a function of a mean intensity of the spectra.

The apparatus of the present invention includes a controllable illumination device for emitting a plurality of electromagnetic radiation wavelengths selected to cause a tissue sample to produce a fluorescence intensity spectrum indicative of tissue abnormality, an optical system for applying the plurality of radiation wavelengths to a tissue sample, a fluorescence intensity spectrum detecting device for detecting an intensity of fluorescence spectra emitted by the sample as a result of illumination by the plurality of electromagnetic

radiation wavelengths, a data processor, connected to the detecting device, for analyzing detected fluorescence spectra to calculate a probability that the sample is abnormal.

These and other features and advantages of the present invention will become apparent to those of ordinary skill in this art with reference to the appended drawings and following detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a graph of the λex/λem values for known cell and tissue flurophores. Also indicated are known cell and tissue fluorophores: first and second harmonic Rayleigh Scattering (1); H_2O Raman Scattering (2); PN (NADH) (3); Fp ($FADH_2$) (4); tryptophan (5); porphyrins (6); collagen (7); elastin (8); Hb absorption bands (9).

FIG. 2 is a graph of the average cell pellet EEM.

FIG. 3 is a graph of a sample cell pellet EEM with a high (430,520) peak.

FIG. 4 is a graph of a sample cell pellet EEM with a high (250,400) peak.

FIG. 5 is a scattergram of cell pellet classification based on principal component analysis of EEM's (spectral data only). (x) indicates samples deemed abnormal by Pap smear reading and (O) indicates samples deemed normal by Pap smear reading; 44 total = 15 normal and 29 abnormal.

FIG. 6 is a graph showing classification errors for Fisher's discriminant analysis (● - cross validation error estimates; ■ - Fisher's method errors).

FIG. 7 is a graph of typical ThinPrep *versus* Pellet Spectra for 280 nm excitation wavelength (--- is thin prep slide; — is cell pellet).

FIG. 8 is a graph of typical ThinPrep *versus* Pellet Spectra for 370 nm excitation wavelength (--- is thin prep slide; — is cell pellet).

FIG. 9 is a graph of a hypothetical distribution of test values for hypothetical samples (1 is specificity; 2 is sensitivity).

DETAILED DESCRIPTION

I. Introduction

Clinical detection of neoplasias can be divided into two different kinds of analysis. First, screening provides a way to identify suspicious samples taken from a rather large pool of subjects. Subjects may be from the population as a whole or they may be part of a group identified as having a higher than average risk for one or more cancers. It is desirable that, because of the sheer number of tests, screening assays be relatively rapid, easy to conduct and inexpensive. It also is desirable that they exhibit a low false-negative rate.

Once patients have been screened, it is necessary to proceed with more detailed testing that can be referred to generically as diagnosis. In diagnosis, the neoplasias nature of the sample is confirmed and, in addition, further information on the type and degree of dysplasia is obtained. This provides the clinician with an understanding of the disease state necessary to begin a treatment regimen. For diagnosis, cost, ease of application and rapidity are less important, though always desirable. It is important, however, that

diagnostic procedures be accurate with respect to the kind of cancer identified and its clinical stage.

The present invention is an example of the first kind of detection, screening. Present screening methods, like Pap smears, are time intensive, require highly trained individuals and are relatively expensive. Even so, the subjective nature of the scoring often results in an unacceptable number of false negatives, the outcome of which can be devastating. It is believed that by using a more objective standard like fluorescent emissions, the accuracy of the screen can be improved. In addition, the possibility for automation has further benefits in terms of time and expense.

A. Method for Determining Fluorescent Spectra

The present invention is premised on the hypothesis that normal and abnormal cells will emit differing fluorescent spectra in response to stimulating electromagnetic radiation. In its most general form, the methods comprises providing a tissue sample, illuminating that tissue sample with electromagnetic radiation, detecting fluorescence of the sample and comparing the fluorescence of the sample with that of some standard. Each of these steps is described in greater detail, below.

Obtaining a tissue sample can be achieved by any one of a variety of different means, largely depending on the nature of the sample to examined. For example, for examination of solid tissues, samples can be taken by biopsy. Alternatively, scrapings of cells can be taken from the tissue of interest. For examination of cells that are not part of solid tissue, liquid samples may be obtained and the cells isolated therefrom. For example, blood samples may be obtained by any normal methodology.

Aspiration of fluids also is contemplated, such as thin tissue aspirates.

Once obtained, it may be necessary to further process the samples before they are examined. Further processing may include various forms of physical arrangement of the samples. For example, with solid tissues, it may be necessary to prepare thin sections. It also may be desired to dissociate the cells from each other and disperse them as a thin film monolayer. Dissociation may be accomplished by physical or enzymatic means. Similarly, dissociated cells in fluid samples or in scrapings may be concentrated and dispersed in a monolayer. In other instances, it may be desirable to concentrate disperse cells as a pellet. This can be accomplished by centrifugation of the liquid samples.

Further pre-illumination processing includes chemical treatments such as fixation steps. In some cases, it will be desirable that the natural autofluorescence of the sample be unaffected. In such a case, the chemical treatment is selected so that the fluorescent species are not altered. In other cases, it may prove useful to use treatments that cause different autofluorescent profiles. Exemplary treatments include alcohol fixation. Suitable alcohols include methanol, ethanol, propanol, isopropanol, n-butanol and t-butanol.

Typically, the samples are provided on a surface, though they can be provided in an open or closed container. A typical surface is a glass or quartz microscope slide. With certain surfaces, such as glass slides, there may be variation from item to item, requiring internal recalibration with each sample. There also may be distorting effects, especially for container-enclosed samples, that must be taken into account.

Once the samples are prepared, the illumination is effected. In the present invention, a variety of different wavelengths can be used spanning from about 200 nm to over 700 nm. Under most circumstances, a plurality of different wavelengths will be applied, individually, to a single sample. Generally, the greater the number of wavelengths used, the better the ability to discriminate between physiologically distinct tissue samples. Of course, at some point, the intervals between wavelengths will be so small that the information achieved will become redundant. Those of skill in the art, knowing the fluorescent behavior of biological molecules, will be able to select both the appropriate number and range of wavelengths for a given purpose.

In one embodiment, wavelengths from 250 nm to 550 nm were applied, with 10 nm intervals. Thus, 31 different wavelengths were applied to a single sample in sequence. Because a series of different illuminations and emissions are required, it is important that the sample not be affected by the illuminating wavelengths. One such effect would be photobleaching, which has been shown to be significant in arterial tissue above excitation fluences of 80 mJ/mm^2.

The next step in the method is detection. For each illuminating wavelength, an emission spectrum is determined over a range of wavelengths. Again, a series of wavelengths may be used with the greater number examined, the more information with which to detect differences between normal and abnormal tissues. The emission spectra are normalized to an appropriate $\lambda ex/\lambda em$. The excitation-emission matrices (EEM's) may be plotted three dimensionally, with excitation wavelength, emission wavelength and $\log(1/1(\lambda ex/\lambda em))$ as the three axes. FIG. 2, FIG. 3, and FIG. 4 depict various EEM's.

In another embodiment, emissions were monitored from 250 nm to 700 nm at 10 nm intervals. Thus, the spectra comprises 46 different emission readings. These readings were normalized to an λex/λem of (270,330). Based on the results discussed below, it appears that individual wavelengths do not adequately discriminate between normal and abnormal tissues. Taken as a group, however, there appears to be a correlation between fluorescence values and pathology. In order to maximize this correlation, various statistical manipulations were applied to the data, as discussed in detail below.

FIG. 1 is a graph showing the excitation/emission profiles for various known cell and tissue fluorophores. Those of skill in the art are aware of other potential natural fluorophores whose fluorescence may be used to generate emission spectra which may then be used in accordance with the present invention.

B. <u>Multi-Variate Statistical Method Development</u>

In order to maximize the correlation between fluorescence values and the physiologic state of the sample tissue, multi-variate statistics were applied. The five primary steps involved in the multivariate statistical method are 1) preprocessing of spectral data from each patient to account for interpatient variation, 2) dimension reduction of the preprocessed spectra in the calibration set using principal component analysis, 3) selection of the diagnostically most useful principal components using a two-sided unpaired t-test and other criteria and 4) development of an optimal classification scheme based on Fisher's discriminant analysis using the diagnostically useful principal component scores of the calibration set as inputs with cross-validation. These five individual steps of the multivariate statistical method are presented below in more detail.

1) **Preprocessing:** The objective of preprocessing is to calibrate tissue spectra for inter-patient variation which might obscure differences in the spectra of different tissue types. A normalization method of preprocessing was invoked on the spectral data.

Spectra were normalized by dividing the fluorescence intensity at each emission wavelength by the fluorescence intensity at (280 nm, 330 nm) of that sample. Normalizing a fluorescence spectrum removes absolute intensity information; methods developed from normalized fluorescence spectra rely on differences in spectral line shape information for diagnosis.

2) **Principal Component Analysis:** Principal component analysis (PCA) is a linear model which transforms the original variables of a fluorescence emission spectrum into a smaller set of linear combinations of the original variables called principal components that account for most of the variance of the original data set. Principal component analysis is described in Dillon W.R., Goldstein M., *Multivariate Analysis: Methods and Applications*, John Wiley and Sons, 1984, pp. 23-52, the disclosure of which is expressly incorporated herein by reference. While PCA may not provide direct insight to the morphologic and biochemical basis of tissue spectra, it provides a novel approach of condensing all the spectral information into a few manageable components, with minimal information loss. Furthermore, each principal component can be easily related to the original emission spectrum, thus providing insight into diagnostically useful emission variables.

Prior to PCA, a data matrix is created where each row of the matrix contains the concatenated preprocessed fluorescence spectra of a sample and each column contains the pre-processed fluorescence intensity at each

excitation-emission wavelength pair. The data matrix D (r x c), consisting of r rows (corresponding to r total samples from all patients in the training set) and c columns (corresponding to intensity at c emission-excitation wavelength pairs) can be written as:

$$D = \begin{pmatrix} D_{11}\ D_{12}\ldots D_{1c} \\ D_{21}\ D_{22}\ldots D_{2c} \\ \\ D_{r1}\ D_{r2}\ldots D_{rc} \end{pmatrix} \qquad (1)$$

The first step in PCA is to calculate the covariance matrix, Z. First, each column of the preprocessed data matrix D is mean-scaled. The mean-scaled preprocessed data matrix, D_m is then multiplied by its transpose and each element of the resulting square matrix is divided by (r-1), where r is the total number of samples. The equation for calculating Z is defined as:

$$Z = \frac{1}{r-1}(D_m/\ D_m) \qquad (2)$$

The square covariance matrix, Z (c x c) is decomposed into its respective eigenvalues and eigenvectors. Because of experimental error, the total number of eigenvalues will always equal the total number of columns (c) in the data matrix D assuming that c < r. The goal is to select n < c eigenvalues that can describe most of

the variance of the original data matrix to within experimental error. The variance, V, accounted for by the first n eigenvalues can be calculated as follows:

$$V = 100\left(\frac{\sum_{j=1}^{n}\lambda_j}{\sum_{j=1}^{c}\lambda_j}\right) \tag{3}$$

The criterion used in this analysis was to retain the first n eigenvalues and corresponding eigenvectors that account for 99.9 % of the variance in the original data set.

Next, the principal component score matrix can be calculated according to the following equation:

$$R = D\,C \tag{4}$$

where, D (r x c) is the preprocessed data matrix and C (c x n) is a matrix whose columns contain the n eigenvectors which correspond to the first n eigenvalues. Each row of the score matrix R (r x c) corresponds to the principal component scores of a sample and each column corresponds to a principal component. The principal components are mutually orthogonal to each other.

Finally, the component loading is calculated for each principal component. The component loading represents the correlation between the principal component and the variables of the original fluorescence emission spectrum. The component loading can be calculated as shown below:

$$CL_{ij} = \frac{C_{ij}}{\sqrt{S_{ii}}}\sqrt{\lambda_j} \qquad (5)$$

where, CL_{ij} represents the correlation between the ith variable (preprocessed intensity at ith emission wavelength) and the jth principal component. C_{ij} is the ith component of the jth eigenvector, λ_j is the jth eigenvalue and S_{ii} is the variance of the ith variable.

Principal component analysis was performed on each type of preprocessed data matrix, described above. Eigenvalues accounting for 99.9% of the variance in the original preprocessed data set were retained The corresponding eigenvectors were then multiplied by the original data matrix to obtain the principal component score matrix R.

3) Student's T-Test: Average values of principal component scores were calculated for each histopathologic tissue category for each principal component obtained from the preprocessed data matrix. A two-sided unpaired student's t-test was employed to determine the diagnostic contribution of each principal component. Such a test is disclosed in Devore J.L., *Probability and Statistics for Engineering and the Sciences*, Brooks/Cole, 1992, and in Walpole R.E., Myers R.H., *Probability and Statistics for Engineers and Scientists*, Macmillan Publishing Co., 1978, Chapter 7, the disclosures of which are expressly incorporated herein by reference. The hypothesis that the means of the principal component scores of two tissue categories are different were tested for normal smears and abnormal smears (ASCUS, LGSIL, HGSIL). Principal components were ranked in order of increasing p value. Fisher's discriminant analysis was performed using the most significant principal components and method

performance was evaluated. Principal components were then added one at a time and Fisher's discriminant analysis was again performed. This process was repeated until no further improvement was reached or a decrease in performance was noted. Principal components chosen in this manner were used in the diagnostic method.

4) Logistic Discrimination: Logistic discriminant analysis is a statistical technique that may be used to develop diagnostic methods based on posterior probabilities, overcoming the drawback of the binary decision scheme employed in the two-stage method. This statistical classification method is based on Bayes theorem and may be used to calculate the posterior probability that an unknown sample belongs to each of the possible tissue categories identified. Logistic discrimination is discussed in Albert A., Harris E.K., *Multivariate Interpretation of Clinical Laboratory Data*, Marcel Dekker, 1987, the disclosure of which is expressly incorporated herein by reference. Classifying the unknown sample into the tissue category for which its posterior probability is highest results in a classification scheme that minimizes the rate of misclassification.

For two diagnostic categories, G_1 and G_2, the posterior probability of being a member of G_1, given measurement x, according to Bayes theorem is:

$$P(G_1|X) = \frac{P(x|G_1)P(G_1)C(2|1)}{P(x|G_1)P(G_1)C(2|1)+P(x|G_2)P(G_2)C(1|2)} \quad (6)$$

where $P(x|G_i)$ is the conditional joint probability that a tissue sample of type i will have principal component score x, and $P(G_i)$ is the prior probability of finding tissue type i in the sample population. $C(j|i)$ is the

cost of misclassifying a sample into group j when the actual membership is group i.

The prior probability $P(G_i)$ is an estimate of the likelihood that a sample of type i belongs to a particular group when no information about it is available. If the sample is considered representative of the population, the observed proportions of cases in each group can serve as estimates of the prior probabilities. In a clinical setting, either historical incidence figures appropriate for the patient population can be used to generate prior probabilities, or the practitioner's colposcopic assessment of the likelihood of precancer can be used to estimate prior probabilities, as is known in the art.

The conditional probabilities may be developed from the probability distributions of the n principal component scores for each tissue type, i. The probability distributions may be modeled using the gamma function, which is characterized by two parameters, alpha and beta, which are related to the mean and standard deviation of the data set. The normal function is typically used to model probability distributions and is defined below:

$$f(x) = (1/(\sqrt{2}\pi)\Sigma)e^{-(x-m/(\sqrt{2})\Sigma)} \tag{7}$$

The normal distribution function may be used to calculate the conditional probability that a sample from tissue type i, will exhibit the principal component score, x. If more than one principal component is needed to describe a sample population, then the conditional joint probability is simply the product of the conditional probabilities of each principal component (assuming that each principal component is an independent variable) for that sample population.

Fisher's discriminant analysis is a particular statistical technique for classifying individuals or objects in to mutually exclusive and exhaustive groups on the basis of a set of independent variables. In this particular application of Fisher's method, the objects are N patient cytological samples, the groups are the diagnostic classifications (normal *versus* abnormal) and the P variables are the principal components X derived from the fluorescence spectra of the samples.

Fisher's method calculates a score Y for each of the samples, based on a linear combination of the variables, *i.e.*,

$$Y(N) = b_1{}^*X_1{}^*(N) + b_2{}^*X_2{}^*(N) + \ldots + b_p{}^*X_p(N)$$

The coefficients b_1 through b_p are calculated so that the difference between the scores for the normals and the abnormals is maximized. Assuming that the X is normally distributed for the two groups, and assuming that the covariance s of X is the same for the two groups, then the best choice for b_1 is

$$b1 = s^{-1*} (\text{avg. of } x_1 \text{ of norm. - avg. of } x_1 \text{ for abnorm.})$$

and similarly for b_2 through b_p. Then, a cutoff value for Y is selected and all samples with scores above the threshold are classified as belonging to the first group, normals, and samples with scores below the threshold are classified as belonging to the second group, abnormals. Since there is overlap in the distributions of Y for the two groups, some samples will be misclassified no matter where the cutoff is chosen. The cutoff is chosen to be the one that results in the lowest misclassification rate. The cutoff value given the above assumptions is

$$Y_c = (n_2Y_1 + n_1Y_2) \ / \ (n_1 + n_2)$$

where n_1 is the number of samples in group 1, and Y_1 is the Y score using the average values of the X variables for group 1, likewise for group 2. Y_c can be adjusted from this value to reduce the FN rate at the expense of the FP rate, or *vice versa*, depending on the application.

Since both the b and Yc values are calculated from the data, it may be asked how well this method will classify new samples, whose values for X were not used in the above-calculations. This performance can be estimated by using cross-validation techniques. For each sample, b and Yc are calculated using the other sample data, and then the method is used to classify that sample. The misclassification error rate for all samples is measured this way is taken as an unbiased estimate of what one can expect when using Fisher's discriminate analysis to classify new samples. Dillon and Goldstein (1985).

II. Examples

In order to evaluate the neoplastic diagnostic potential of cellular autofluorescence, excitation of exfoliated, ethanol-fixed cervical squamous epithelial cells with a plurality of wavelengths was performed. In addition, the effects on the fluorescent spectra of different specimen preparation methods were examined using both cell pellet and monolayer preparations made from the same samples.

A. Sample Preparation

Exfoliated cervical cells were obtained from patients referred to MD Anderson Cancer Center for routine screening, as well as colposcopy, on the basis of previous abnormal cervical cytology. Conventional Pap smears were obtained and the normally discarded cells remaining on the swab suspended in an ethanol-based fixative (PreservCyt Solution, Cytyc Corp., Marlborough,

MA). From each suspension, two types of samples were prepared. First, a monolayer cell touch prep was prepared onto an ordinary glass microscope slide using the CYTYC Thinprep device (Hutchinson, 1992). This device extracts an aliquot from the suspension and filters it to remove red blood cells and other debris smaller and larger than epithelial cells. The cells were deposited in an circular area 20mm in diameter.

The remaining cells in suspension were centrifuged and resuspended three times in HPLC-grade ethanol to remove the fluorescent preservative solution. The number of cells in suspension was determined using a hemocytometer and found to vary between 1 - 50 x 10^4 cells. The cells were then spun down to a pellet, placed on a spot approximately 3 mm in diameter on a quartz microscope slide and air dried.

B. Fluorescent Spectroscopy Apparatus and Conditions

All fluorescence measurements were made using a standard scanning fluorimeter (SPEX, Fluorolog II, Edison, NJ) with a spectral resolution of 5 nm FWHM. Beam area was approximately 2 mm^2. Slides were placed so that the cells were on the side facing the beam, and the beam was focused onto this surface of the slide. Excitation light was incident normally and emission was collected at an angle of 20 degrees from the normal. The signal at each (λex,λem) value was integrated for 2 seconds. Data were corrected for the non-uniform spectral response of the emission monochromator and detector using correction factors supplied with the instrument. Also, spectra were corrected for variability with wavelength in the intensity of the excitation source using a Rhodamine B quantum counter (20).

Fluorescence excitation-emission matrices (EEM's) were recorded for each cell pellet sample. Excitation wavelengths ranged from 250 to 550 nm, in 10 nm increments, and emission was measured from 10 nm above the excitation wavelength to 10 nm below the 2nd harmonic of the excitation wavelength, up to 700 nm, in 10 nm increments. A background EEM was recorded from a quartz slide containing supernatant only from the resuspension of one sample. This background EEM was subtracted from each pellet EEM. For several samples, a second emission spectrum was recorded at 250 nm excitation after the initial EEM to check for photobleaching effects. All EEMs were normalized with respect to the intensity at (270,330).

For the ThinPrep samples, one excitation spectrum from 350 nm to 410 nm at 440 nm emission was recorded, and two emission spectra were recorded, at excitations of 280nm and 370nm, using the same increments and spectral ranges as the EEMs above. Since the UV fluorescence of the glass slides differed between slides, a background spectrum was recorded from each slide from a region with no cells. All spectra were normalized with respect to the intensity at (280,330).

C. Pap Smears

Conventional Pap smears were read by staff cytopathologists at M.D. Anderson Cancer Center. Diagnosis was done using the standard diagnostic classifications, where the samples are classified as Normal, HPV, CIN I (mild dysplasia), CIN II (moderate dysplasia), or CIN III (severe dysplasia or carcinoma in situ), and Squamous Carcinoma (Vooijs, 1991).

Samples were obtained from 80 patients, of which 36 were not used due to inconclusive diagnoses, samples too dilute, contamination with blood or other foreign matter

such as cotton from swabs used in the sample collection, or errors made in the data collection. Of the remaining 44 samples, 15 had conventional Pap smears which were diagnosed as normal (negative for malignant cells) and 29 were read as abnormal (HPV - 14; CINI - 9; CINII - 10; CINIII - 10). The ages ranged from 20 to 52, with the average age of 33. There were 21 whites, 7 hispanics, 15 blacks, and 1 oriental. For comparison of the pellet to the ThinPrep preparations, 23 samples were selected from the patients mentioned above, of which 10 were normal and 13 abnormal.

D. **Fixed Cell Fluorescence**

FIG. 2 is a graph of the average EEM for all 44 pellet prepared samples used. All plots are normalized to the (270,330) values and plotted on a log scale. Contour plot lines are spaced evenly also on a log scale. The most intense fluorescence peak is at (280,330), characteristic of tryptophan. A peak also is present at (370,450), and a slight shoulder at (280,450), both characteristic of PN. A shoulder is also present at (250,400), due to contaminants of unknown origin. There is a slight shoulder near (430,520) due to flavoprotein as well as a valley in the excitation spectra corresponding to the Soret Hb absorption line at λex = 415 nm. No significant photobleaching of the cells was observed.

Although the tryptophan and PN peaks were present in all samples, the intensity of the PN and Fp peaks and other features varied greatly among the pellet sample EEM's. FIG. 3 shows an EEM of a sample with the (430,520) peak more pronounced. The Soret absorption band is also clearly visible, while the (250,400) peak is absent completely. For the sample EEM in FIG. 4, the peak at (250,400) is even more intense than the (280,330) tryptophan peak.

Table 1 lists these spectral features, and the average and standard deviation for each. All features showed considerable variation among samples, with the magnitude of the standard deviation exceeding the mean in most cases. The (280,330) peak stability can be attributed to the fact that the spectra were normalized to the nearby (270,330) value. The EEM value with the least percent variance was at (300,360), between the tryptophan and PN peaks.

TABLE 1: SPECTRAL FEATURES OF CELL PELLET EEM's*

feature (λem,λex)	all samples		nor. sample		abn. samples		nor v abn
	μ	σ	μ	σ	μ	σ	p
(280,330)	1.18	0.16	1.16	0.16	1.19	0.17	0.56
(250,400)	0.29	0.61	0.33	0.62	0.28	0.62	0.78
(280,450)	0.08	0.09	0.08	0.09	0.08	0.09	0.93
(370,450)	0.23	0.31	0.21	0.29	0.24	0.33	0.75
(430,520)	0.11	0.13	0.09	0.10	0.12	0.15	0.45
(280,450)/ (370,450)	0.36	0.21	0.40	0.32	0.34	0.28	0.08
(300,360)	0.44	0.13	0.39	0.11	0.46	0.13	0.04

* - all values normalized to intensity at (270,330)

Table 1 also compares population statistics for the normal and abnormal sample groups. For each feature, the variance within each group exceeded the difference between the two groups, as reflected in the high p-values. The lowest p-scores for all values or ratios of values in the EEMs are at the ratio of (280,450)/(370,450) and at (300,360).

In order to test the hypothesis that a combination of several variables may identify significant differences

between normal and abnormal cells, principal component analysis (PCA) was used to generate a reduced set of variables which are linear combinations of the EEM values. From each EEM, 25 principal components (PC's) accounting for 99.99% of the variance between all samples were calculated. None of the PC's had a higher statistical significance than any of the features in Table 1. Table 2 provides this comparison.

TABLE 2

pc#	%VAR	mean-nor	mean-ab	stdev-nor	stdev-ab	p-score
*18	0.0193	0.0207	0.0107	0.0507	0.0324	0.041305
* 5	1.4136	-0.1318	0.0682	0.3042	0.3685	0.063457
* 3	4.3008	-0.2137	0.1105	0.5753	0.6279	0.096108
22	0.0047	0.0050	-0.0026	0.0144	0.0229	0.187096
*16	0.0364	0.0150	-0.0077	0.0491	0.0605	0.188685
23	0.0036	0.0042	-0.0022	0.0169	0.0184	0.25996
*10	0.1573	0.0284	-0.0147	0.1183	0.1191	0.263165
19	0.0106	0.0074	-0.0038	0.0338	0.0292	0.28409
13	0.0627	0.0146	-0.0076	0.0651	0.0801	0.329235
25	0.0023	0.0032	-0.0016	0.0181	0.0120	0.363534
14	0.0463	0.0122	-0.0063	0.0663	0.0641	0.382071
12	0.0801	-0.0139	0.0072	0.0946	0.0806	0.469852
15	0.0442	0.0084	-0.0043	0.0694	0.0605	0.553599
8	0.3687	-0.0211	0.0109	0.1569	0.1962	0.561494
7	0.5198	0.0243	-0.0126	0.2173	0.2192	0.599062
1	66.9477	-0.2033	0.1052	2.1941	2.6173	0.682091
21	0.0062	0.0019	-0.0010	0.0226	0.0246	0.695786
17	0.0224	0.0039	-0.0020	0.0534	0.0408	0.707169
9	0.2596	-0.0104	0.0054	0.1533	0.1555	0.75051

pc#	%VAR	mean-nor	mean-ab	sidev-nor	sidev-ab	p-score
20	0.0079	-0.0013	0.0007	0.0233	0.0288	0.800828
24	0.0031	-0.0006	0.0003	0.0196	0.0156	0.876723
2	22.881	-0.0348	0.0180	1.4811	1.4414	0.910808
4	1.9629	0.0080	-0.0042	0.4774	0.3980	0.933139

pc#	%VAR	mean-nor	mean-ab	stdev-nor	stdev-ab	p-score
6	0.7267	0.0039	-0.0020	0.2047	0.2826	0.936508
11	0.112	0.0005	-0.0003	0.1313	0.0832	0.983305

* used in Dx algorithm
total %VAR used = 5.927%

A discriminant function was found using Fisher's discriminant analysis (FDA), assuming equal prior probabilities and variable cost of misclassification. This function used 5 of the PCs above which together account for 5.93% of the sample-to-sample variance (3,5,10,16,18). The discriminant score calculated for each sample is plotted in FIG. 5, where samples above Y_c are classified as normal, samples below Y_c abnormals. The Pap smear diagnosis is shown as well (o - normals, x - abnormals). The false positive rate (FP) is 2/15 = 13.4%, while the false negative rate (FN) is 12/29 = 41.4%, where the Y_c is 0 (solid line). Another choice is Y_c is 0.6, where the FN is 4/29 = 13.8% and the FP is 5/15 = 33.3% (broken line). A plot of the FN versus FP, obtained by varying Y_c in FIG. 5, is shown in FIG. 6, with the diagonal line representing random classification. The expected performance of the discriminant function on additional samples was estimated using cross validation, with the results shown in FIG. 6.

E. <u>ThinPrep vs Pellet Autofluorescence</u>

FIG. 7 compares ThinPrep and pellet emission spectra from a typical sample for 280 nm excitation. For all samples, the spectra are similar in shape, except that the ThinPrep spectra are blue shifted by an average of 10 nm. In FIG. 8, the spectra at 370 nm excitation are compared for a typical case. Again, the ThinPrep spectrum is blue-shifted with respect to the pellet spectrum. In addition, the intensity at the ThinPrep PN peak around (370,450) is 5- to 10-times lower than the

corresponding pellet spectra. The variance between samples is reduced for the ThinPrep spectra, having a lower standard deviation as a percentage of the mean, 58%, compared to 82% for the pellet data. The excitation spectra of the ThinPrep slides recorded at 440 nm emission showed considerable variance below 350 nm excitation due to the strong UV emission of the glass slides. Above 350 nm excitation, the spectra differed from the pellet spectra in the degree of variance between samples and in the intensity, as described above for the (370,450) peak.

APPENDIX I: SPECIFICITY AND SENSITIVITY

Summarized from: Albert A., Harris E.K.: *Multivariate Interpretation of Clinical Laboratory Data*, Marcel Dekker Inc., New York, pp. 75-82, (1987), the disclosure of which is expressly incorporated herein by reference.

Assuming a group of T samples which can be categorized as normal (N samples) or diseased (D samples). A diagnostic test, designed to determine whether the sample is normal or diseased, is applied to each sample. The results of the tests is the continuous variable x, which is then used to determine the sample type. FIG. 9 illustrates a hypothetical distribution of test values for each sample type. A diagnostic method based on this test can easily be defined by choosing a cutoff point, d, such that a sample with an observed value $x<d$ is diagnosed as normal and a sample with an observed value $x \geq d$ is diagnosed as abnormal.

Several quantitative measures have been defined to 'evaluate' the performance of this type of method. The first type evaluates the test itself (*i.e.*, measures the ability of the test to separate the two populations, N and D). Sensitivity and specificity are two such measures. The second type is designed to aid in the interpretation of a particular test result (i.e. deciding whether the individual test measurement has come from a normal or diseased sample). Positive and negative predictive value are two measures of this type.

To define these measures, some terminology and notation must be introduced. Referring to Table 3, a sample to be tested can be either normal or diseased; the result of the test for each type of sample can be either negative or positive. True negatives represent those normal with a positive test result. In these cases, the

diagnosis based on the rest result is correct. False positives are those normal samples which have a positive test result and false negatives are those diseased samples which have a negative test result. In these cases, the diagnosis based on the test result is incorrect.

TABLE 3

	Normal	**Diseased**	**Total Samples**
Test Negative ($x<d$)	True Negatives (TN)	False Negatives (FN)	Negatives (Neg)
Test Positive ($x\geq d$)	False Positives (FP)	True Positives (TP)	Positives (Pos)
Total Samples	N	D	T

With this terminology, Table 4 contains a definition of sensitivity and specificity, the two measures which assess the performance of the diagnostic method. Specificity is the proportion of normal samples with a negative test result (proportion of normal samples diagnosed correctly). Sensitivity is the proportion of diseased samples with a positive test result (Proportion of diseased samples correctly diagnosed). FIG. 9 also contains a graphical representation of specificity and sensitivity. Specificity represents the area under the normal sample distribution curve to the left of the cut off point while sensitivity represent the area under the diseased sample distribution curve to the right of the cut off point.

TABLE 4

Test Measure	Meaning	Calculation
Specificity	Proportion of normal samples with negative test result	Sp=TN/N
Sensitivity	Proportion of diseased samples with positive test result	Se=TP/D

While sensitivity and specificity characterize the performance of a particular method, another set of statistics is required to interpret the laboratory test result for a given specimen. The positive and negative predictive value quantify the meaning of an individual test result (Table 5). The positive predictive value is the probability that if the test result is positive, the sample is diseased. The negative predictive value is the probability that if the test result is negative, the sample is normal. Positive and negative predictive value are calculated from Baye's rule as outlined in Albert and Harris. Table 5 contains two equivalent formulas for calculation positive and negative predictive value.

TABLE 5

Measure	Meaning	Calculation 1	Calculation 2
Positive Predictive Value	The probability that, if the test is positive, the sample is diseased	$PV_{+}=TP/Pos$	$PV_{+}=DSe/(DSe+N(1-Sp))$
Negative Predictive Value	The probability that, if the test is negative, the sample is normal	$PV_{-}=TN/Neg$	$PV_{-}=NSp/(NSp+D(1-Se))$

APPENDIX II: PRINCIPAL COMPONENTS

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
250	280	0.155	0.353	-0.031	-0.058	-0.055
250	290	0.058	0.097	-0.020	0.077	0.109
250	300	0.024	0.055	-0.033	0.035	0.096
250	310	0.028	0.106	-0.023	0.064	0.186
250	320	0.037	0.153	0.000	0.119	0.168
250	330	0.016	0.188	-0.001	0.052	0.034
250	340	-0.004	0.197	-0.012	-0.003	-0.064
250	350	-0.014	0.187	-0.046	-0.030	-0.140
250	360	-0.015	0.132	-0.051	-0.029	-0.134
250	370	-0.011	0.060	-0.040	-0.010	-0.110
250	380	-0.004	-0.017	-0.013	0.012	-0.039
250	390	-0.003	-0.069	0.019	0.021	-0.010
250	400	-0.005	-0.088	0.049	-0.001	0.010
250	410	-0.010	-0.082	0.051	-0.009	0.006
250	420	-0.012	-0.061	0.046	-0.008	0.061
250	430	-0.013	-0.047	0.044	-0.006	0.059
250	440	-0.013	-0.031	0.038	0.003	0.063
250	450	-0.013	-0.022	0.041	-0.001	0.060
250	460	-0.010	-0.014	0.034	0.006	0.054
260	290	-0.013	0.017	-0.063	-0.046	-0.101
260	300	-0.030	-0.026	-0.102	-0.134	-0.044
260	310	-0.009	0.007	-0.093	-0.019	0.085
260	320	-0.003	0.035	-0.078	0.054	0.160

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
260	330	-0.029	0.060	-0.091	0.036	0.052
260	340	-0.045	0.085	-0.121	-0.009	-0.008
260	350	-0.046	0.087	-0.143	-0.031	-0.088
260	360	-0.037	0.066	-0.136	-0.004	-0.119
260	370	-0.029	0.042	-0.115	-0.005	-0.094
260	380	-0.023	0.015	-0.077	-0.006	-0.088
260	390	-0.021	-0.007	-0.039	-0.011	-0.062
260	400	-0.021	-0.021	-0.010	-0.022	-0.048
260	410	-0.023	-0.025	0.010	-0.030	-0.039
260	420	-0.023	-0.023	0.022	-0.031	-0.009
260	430	-0.022	-0.023	0.031	-0.027	-0.024
260	440	-0.021	-0.020	0.037	-0.029	-0.026
260	450	-0.019	-0.018	0.046	-0.028	-0.026
260	460	-0.016	-0.015	0.044	-0.024	-0.032
260	470	-0.014	-0.014	0.044	-0.023	-0.027
260	480	-0.012	-0.011	0.039	-0.020	-0.020
270	300	-0.032	-0.105	-0.059	0.017	-0.173
270	310	0.008	-0.068	-0.036	-0.005	-0.040
270	320	0.026	-0.035	-0.014	0.006	0.028
270	330	0.000	0.000	0.000	0.000	0.000
270	340	-0.020	0.038	-0.025	0.011	0.002
270	350	-0.026	0.046	-0.058	0.073	-0.024
270	360	-0.021	0.035	-0.062	0.124	-0.039
270	370	-0.017	0.017	-0.058	0.140	-0.048

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
270	380	-0.016	0.004	-0.036	0.115	-0.042
270	390	-0.020	-0.005	-0.009	0.086	-0.035
270	400	-0.024	-0.012	0.011	0.062	-0.038
270	410	-0.030	-0.016	0.027	0.046	-0.042
270	420	-0.031	-0.014	0.040	0.035	-0.011
270	430	-0.031	-0.016	0.051	0.035	-0.038
270	440	-0.030	-0.015	0.059	0.028	-0.040
270	450	-0.027	-0.015	0.066	0.022	-0.042
270	460	-0.023	-0.015	0.064	0.018	-0.048
270	470	-0.020	-0.013	0.060	0.013	-0.048
270	480	-0.017	-0.012	0.055	0.011	-0.037
270	490	-0.015	-0.012	0.048	0.012	-0.024
270	500	-0.013	-0.011	0.042	0.011	-0.008
280	310	0.168	-0.238	-0.057	0.027	-0.099
280	320	0.225	-0.213	-0.037	0.000	-0.067
280	330	0.223	-0.196	-0.046	0.007	-0.052
280	340	0.200	-0.159	-0.089	0.063	-0.006
280	350	0.161	-0.124	-0.136	0.113	0.008
280	360	0.118	-0.089	-0.137	0.126	0.033
280	370	0.079	-0.065	-0.122	0.104	0.028
280	380	0.045	-0.048	-0.091	0.073	0.024
280	390	0.020	-0.040	-0.049	0.042	0.014
280	400	-0.001	-0.035	-0.022	0.024	0.006
280	410	-0.014	-0.030	0.001	0.009	0.011

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
280	420	-0.020	-0.023	0.018	-0.004	0.031
280	430	-0.022	-0.022	0.031	0.001	0.000
280	440	-0.023	-0.021	0.041	-0.002	-0.009
280	450	-0.021	-0.020	0.048	0.000	-0.012
280	460	-0.018	-0.019	0.051	0.001	-0.024
280	470	-0.016	-0.018	0.049	0.000	-0.031
280	480	-0.014	-0.017	0.046	0.001	-0.032
280	490	-0.012	-0.016	0.042	0.002	-0.018
280	500	-0.010	-0.014	0.037	0.006	-0.011
280	510	-0.009	-0.013	0.032	0.006	-0.005
280	520	-0.008	-0.012	0.029	0.003	-0.005
290	320	0.348	-0.068	0.019	-0.279	0.024
290	330	0.363	-0.066	0.015	-0.231	-0.020
290	340	0.335	-0.053	-0.048	-0.104	0.010
290	350	0.278	-0.040	-0.113	-0.007	0.012
290	360	0.204	-0.029	-0.137	0.057	0.023
290	370	0.140	-0.027	-0.129	0.065	0.017
290	380	0.083	-0.028	-0.097	0.041	0.002
290	390	0.044	-0.030	-0.064	0.015	0.000
290	400	0.016	-0.032	-0.034	-0.007	0.002
290	410	-0.002	-0.030	-0.015	-0.020	0.007
290	420	-0.010	-0.024	0.002	-0.033	0.030
290	430	-0.012	-0.024	0.014	-0.028	0.006
290	440	-0.013	-0.023	0.023	-0.027	-0.005

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
290	450	-0.012	-0.023	0.033	-0.021	-0.012
290	460	-0.010	-0.023	0.036	-0.016	-0.026
290	470	-0.009	-0.022	0.036	-0.013	-0.027
290	480	-0.007	-0.020	0.036	-0.011	-0.025
290	490	-0.006	-0.019	0.034	-0.007	-0.024
290	500	-0.005	-0.019	0.030	-0.003	-0.021
290	510	-0.004	-0.018	0.028	-0.001	-0.018
290	520	-0.004	-0.017	0.026	-0.001	-0.012
290	530	-0.003	-0.016	0.024	-0.004	-0.011
290	540	-0.003	-0.015	0.022	-0.003	-0.007
300	330	0.230	0.211	0.331	-0.028	0.004
300	340	0.221	0.226	0.276	0.058	-0.019
300	350	0.184	0.212	0.168	0.105	-0.024
300	360	0.136	0.171	0.081	0.104	-0.021
300	370	0.093	0.125	0.021	0.075	-0.019
300	380	0.054	0.077	-0.006	0.030	-0.008
300	390	0.027	0.041	-0.015	-0.007	-0.005
300	400	0.007	0.012	-0.017	-0.035	-0.006
300	410	-0.005	-0.002	-0.017	-0.049	-0.008
300	420	-0.010	-0.007	-0.010	-0.057	0.015
300	430	-0.010	-0.012	-0.005	-0.048	-0.010
300	440	-0.009	-0.013	0.001	-0.045	-0.013
300	450	-0.007	-0.015	0.010	-0.037	-0.015
300	460	-0.005	-0.016	0.014	-0.028	-0.026

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
300	470	-0.003	-0.016	0.017	-0.024	-0.031
300	480	-0.002	-0.016	0.019	-0.019	-0.028
300	490	0.000	-0.016	0.019	-0.013	-0.026
300	500	0.000	-0.016	0.019	-0.008	-0.021
300	510	0.001	-0015	0.018	-0.006	-0.024
300	520	0.001	-0.015	0.018	-0.006	-0.018
300	530	0.001	-0.014	0.018	-0.007	-0.018
300	540	0.001	-0.013	0.016	-0.007	-0.015
300	550	0.001	-0.012	0.016	-0.007	-0.015
300	560	0.001	-0.010	0.013	-0.007	-0.015
310	340	0.004	0.052	0.029	-0.010	-0.021
310	350	-0.002	0.058	-0.003	-0.035	-0.030
310	360	-0.006	0.051	-0.026	-0.055	-0.010
310	370	-0.009	0.039	-0.042	-0.073	-0.003
310	380	-0.012	0.025	-0.047	-0.084	0.020
310	390	-0.013	0.012	-0.043	-0.088	0.025
310	400	-0.015	0.000	-0.042	-0.080	0.008
310	410	-0.017	-0.007	-0.039	-0.077	-0.002
310	420	-0.017	-0.007	-0.029	-0.073	0.017
310	430	-0.014	-0.012	-0.023	-0.054	-0.019
310	440	-0.012	-0.011	-0.016	-0.047	-0.025
310	450	-0.008	-0.012	-0.006	-0.036	-0.024
310	460	-0.004	-0.013	0.001	-0.024	-0.037
310	470	-0.002	-0.012	0.004	-0.016	-0.038

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
330	470	0.002	-0.018	0.001	0.020	-0.038
330	480	0.003	-0.016	0.004	0.023	-0.028
330	490	0.005	-0.015	0.005	0.029	-0.025
330	500	0.006	-0.014	0.006	0.031	-0.024
330	510	0.006	-0.013	0.006	0.030	-0.020
330	520	0.006	-0.012	0.007	0.028	-0.013
330	530	0.005	-0.011	0.009	0.023	-0.014
330	540	0.005	-0.010	0.009	0.020	-0.013
330	550	0.004	-0.009	0.008	0.016	-0.012
330	560	0.004	-0.007	0.007	0.013	-0.012
330	570	0.004	-0.006	0.005	0.011	-0.009
330	580	0.003	-0.006	0.005	0.008	-0.013
330	590	0.003	-0.004	0.003	0.006	-0.011
330	600	0.003	-0.003	0.002	0.006	-0.011
330	610	0.002	-0.003	0.002	0.005	-0.010
330	620	0.002	-0.002	0.001	0.005	-0.009
340	370	-0.005	0.005	-0.032	-0.029	0.062
340	380	-0.007	0.004	-0.035	-0.030	0.114
340	390	-0.009	-0.001	-0.029	-0.025	0.130
340	400	-0.011	-0.011	-0.034	-0.006	0.063
340	410	-0.015	-0.018	-0.042	0.005	0.001
340	420	-0.015	-0.017	-0.023	0.007	0.049
340	430	-0.010	-0.024	-0.020	0.039	-0.041
340	440	-0.006	-0.023	-0.012	0.042	-0.036

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
340	450	-0.001	-0.023	0.007	0.048	-0.020
340	460	0.004	-0.023	0.010	0.057	-0.044
340	470	0.006	-0.020	0.012	0.051	-0.039
340	480	0.008	-0.017	0.016	0.050	-0.022
340	490	0.009	-0.016	0.016	0.050	-0.022
340	500	0.009	-0.014	0.014	0.048	-0.013
340	510	0.009	-0.012	0.013	0.043	-0.006
340	520	0.009	-0.011	0.013	0.039	-0.004
340	530	0.008	-0.010	0.013	0.032	-0.002
340	540	0.007	-0.008	0.012	0.026	-0.004
340	550	0.006	-0.007	0.011	0.021	-0.003
340	560	0.005	-0.006	0.010	0.016	-0.006
340	570	0.005	-0.005	0.007	0.013	-0.007
340	580	0.004	-0.004	0.005	0.012	-0.008
340	590	0.004	-0.003	0.004	0.009	-0.007
340	600	0.003	-0.002	0.002	0.007	-0.008
340	610	0.003	-0.002	0.002	0.007	-0.009
340	620	0.003	-0.001	0.002	0.006	-0.010
340	630	0.003	0.000	0.001	0.006	-0.011
340	640	0.002	0.000	0.001	0.004	-0.011
350	380	-0.004	0.003	-0.025	0.007	0.119
350	390	-0.005	-0.002	-0.016	0.010	0.154
350	400	-0.007	-0.011	-0.031	0.034	0.082
350	410	-0.013	-0.019	-0.047	0.038	0.016

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
310	480	0.000	-0.012	0.007	-0.009	-0.037
310	490	0.001	-0.012	0.009	-0.003	-0.035
310	500	0.002	-0.012	0.009	0.002	-0.032
310	510	0.003	-0.011	0.011	0.004	-0.027
310	520	0.003	-0.011	0.011	0.004	-0.024
310	530	0.003	-0.011	0.013	0.003	-0.023
310	540	0.003	-0.010	0.012	0.002	-0.022
310	550	0.003	-0.009	0.011	0.001	-0.018
310	560	0.003	-0.008	0.009	0.002	-0.016
310	570	0.002	-0.007	0.008	0.001	-0.016
310	580	0.002	-0.006	0.006	0.000	-0.014
320	350	-0.019	0.017	-0.041	-0.101	-0.022
320	360	-0.020	0.015	-0.054	-0.129	0.000
320	370	-0.020	0.013	-0.060	-0.141	0.011
320	380	-0.020	0.007	-0.059	-0.138	0.040
320	390	-0.019	0.000	-0.053	-0.125	0.045
320	400	-0.018	-0.008	-0.047	-0.101	0.023
320	410	-0.019	-0.014	-0.045	-0.084	0.004
320	420	-0.018	-0.013	-0.034	-0.071	0.028
320	430	-0.013	-0.017	-0.029	-0.044	-0.018
320	440	-0.010	-0.016	-0.022	-0.031	-0.025
320	450	-0.006	-0.016	-0.010	-0.018	-0.025
320	460	-0.002	-0.017	-0.005	-0.005	-0.040
320	470	0.000	-0.015	-0.001	0.001	-0.041

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
320	480	0.002	-0.013	0.003	0.005	-0.036
320	490	0.003	-0.013	0.005	0.013	-0.035
320	500	0.004	-0.012	0.005	0.016	-0.030
320	510	0.004	-0.012	0.007	0.017	-0.026
320	520	0.004	-0.011	0.008	0.016	-0.024
320	530	0.004	-0.010	0.009	0.014	-0.021
320	540	0.004	-0.009	0.009	0.011	-0.023
320	550	0.004	-0.008	0.008	0.010	-0.020
320	560	0.003	-0.008	0.007	0.008	-0.018
320	570	0.003	-0.007	0.006	0.007	-0.016
320	580	0.003	-0.006	0.005	0.005	-0.014
320	590	0.002	-0.005	0.004	0.004	-0.015
320	600	0.002	-0.004	0.003	0.004	-0.012
330	360	-0.015	0.013	-0.046	-0.096	0.010
330	370	-0.016	0.011	-0.053	-0.109	0.031
330	380	-0.016	0.007	-0.053	-0.107	0.071
330	390	-0.016	-0.001	-0.046	-0.099	0.076
330	400	-0.016	-0.010	-0.046	-0.075	0.036
330	410	-0.018	-0.017	-0.047	-0.058	0.000
330	420	-0.017	-0.016	-0.033	-0.049	0.033
330	430	-0.013	-0.022	-0.029	-0.017	-0.028
330	440	-0.009	-0.021	-0.022	-0.006	-0.028
330	450	-0.005	-0.020	-0.008	0.004	-0.022
330	460	-0.001	-0.021	-0.003	0.017	-0.042

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
350	420	-0.016	-0.017	-0.022	0.029	0.088
350	430	-0.010	-0.027	-0.016	0.071	-0.028
350	440	-0.006	-0.025	-0.007	0.065	-0.005
350	450	0.001	-0.026	0.017	0.070	0.019
350	460	0.007	-0.027	0.021	0.078	-0.008
350	470	0.009	-0.023	0.020	0.068	0.003
350	480	0.011	-0.019	0.024	0.062	0.022
350	490	0.012	-0.017	0.022	0.062	0.023
350	500	0.012	-0.015	0.018	0.059	0.028
350	510	0.011	-0.012	0.015	0.051	0.035
350	520	0.011	-0.011	0.014	0.043	0.035
350	530	0.009	-0.009	0.013	0.036	0.029
350	540	0.008	-0.007	0.012	0.028	0.026
350	550	0.008	-0.006	0.009	0.022	0.023
350	560	0.006	-0.004	0.007	0.017	0.016
350	570	0.006	-0.003	0.006	0.013	0.012
350	580	0.005	-0.003	0.004	0.010	0.007
350	590	0.004	-0.002	0.002	0.008	0.004
350	600	0.004	-0.001	0.001	0.007	0.003
350	610	0.004	-0.001	0.000	0.006	-0.001
350	620	0.003	0.000	0.000	0.005	-0.005
350	630	0.003	0.001	0.000	0.005	-0.004
350	640	0.003	0.001	0.000	0.004	-0.007
350	650	0.003	0.001	-0.001	0.004	-0.008

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
350	660	0.002	0.001	-0.001	0.004	-0.009
360	390	-0.005	-0.002	-0.004	0.012	0.131
360	400	-0.008	-0.012	-0.025	0.033	0.051
360	410	-0.016	-0.016	-0.058	0.025	-0.019
360	420	-0.021	-0.012	-0.026	0.007	0.093
360	430	-0.013	-0.025	-0.017	0.059	-0.039
360	440	-0.010	-0.021	-0.009	0.051	-0.010
360	450	-0.001	-0.024	0.024	0.062	0.035
360	460	0.007	-0.028	0.032	0.079	0.002
360	470	0.010	-0.022	0.030	0.067	0.015
360	480	0.012	-0.018	0.034	0.061	0.043
360	490	0.014	-0.017	0.031	0.063	0.050
360	500	0.014	-0.014	0.023	0.060	0.054
360	510	0.013	-0.011	0.018	0.050	0.064
360	520	0.012	-0.009	0.015	0.042	0.062
360	530	0.011	-0.007	0.013	0.034	0.058
360	540	0.010	-0.006	0.010	0.026	0.050
360	550	0.008	-0.004	0.007	0.020	0.045
360	560	0.007	-0.003	0.005	0.015	0.034
360	570	0.006	-0.002	0.004	0.011	0.027
360	580	0.006	-0.001	0.001	0.008	0.022
360	590	0.005	-0.001	0.000	0.005	0.015
360	600	0.004	0.000	-0.001	0.004	0.010
360	610	0.004	0.001	-0.002	0.005	0.06

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
360	620	0.003	0.001	-0.001	0.004	0.002
360	630	0.003	0.002	-0.001	0.004	-0.001
360	640	0.003	0.002	-0.001	0.004	-0.003
360	650	0.003	0.002	-0.002	0.005	-0.005
360	660	0.003	0.002	-0.002	0.004	-0.006
360	670	0.002	0.002	-0.002	0.005	-0.008
360	680	0.002	0.002	-0.002	0.004	-0.007
370	400	-0.007	-0.011	-0.031	0.021	-0.018
370	410	-0.017	-0.013	-0.073	-0.002	-0.093
370	420	-0.024	-0.005	-0.039	-0.038	0.052
370	430	-0.016	-0.021	-0.024	0.022	-0.085
370	440	-0.014	-0.016	-0.010	0.010	-0.044
370	450	-0.004	-0.020	0.030	0.026	0.005
370	460	0.007	-0.026	0.042	0.056	-0.023
370	470	0.010	-0.020	0.041	0.047	0.002
370	480	0.012	-0.016	0.044	0.044	0.038
370	490	0.015	-0.015	0.039	0.051	0.045
370	500	0.015	-0.012	0.029	0.049	0.058
370	510	0.014	-0.009	0.023	0.042	0.074

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
370	520	0.013	-0.007	0.017	0.033	0.073
370	530	0.011	-0.005	0.013	0.026	0.068
370	540	0.010	-0.003	0.009	0.018	0.061
370	550	0.009	-0.001	0.005	0.014	0.053
370	560	0.008	0.000	0.002	0.008	0.045
370	570	0.007	0.000	0.001	0.006	0.038
370	580	0.006	0.001	-0.002	0.003	0.028
370	590	0.005	0.001	-0.002	0.001	0.023
370	600	0.004	0.002	-0.003	0.002	0.017
370	610	0.004	0.002	-0.003	0.002	0.011
370	620	0.004	0.003	-0.004	0.001	0.007
370	630	0.004	0.002	-0.004	0.002	0.002
370	640	0.003	0.003	-0.003	0.002	-0.003
370	650	0.003	0.003	-0.003	0.002	-0.003
370	660	0.003	0.003	-0.003	0.003	-0.005
370	670	0.002	0.003	-0.003	0.003	-0.006
370	680	0.002	0.003	-0.003	0.003	-0.006
370	690	0.002	0.003	-0.003	0.003	-0.007
380	410	-0.017	-0.003	-0.088	-0.035	-0.123
380	420	-0.026	0.008	-0.053	-0.089	0.040
380	430	-0.017	-0.010	-0.034	-0.030	-0.080
380	440	-0.017	-0.002	-0.021	-0.049	-0.043
380	450	-0.007	-0.008	0.028	-0.028	0.004
380	460	0.005	-0.017	0.044	0.012	-0.023

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
380	470	0.008	-0.011	0.042	0.008	0.002
380	480	0.011	-0.008	0.047	0.012	0.033
380	490	0.014	-0.009	0.045	0.025	0.040
380	500	0.014	-0.007	0.031	0.025	0.058
380	510	0.013	-0.004	0.024	0.020	0.071
380	520	0.012	-0.003	0.019	0.017	0.074
380	530	0.011	-0.002	0.014	0.013	0.070
380	540	0.010	0.000	0.009	0.007	0.063
380	550	0.008	0.001	0.005	0.002	0.056
380	560	0.007	0.002	0.002	-0.001	0.048
380	570	0.006	0.003	0.000	-0.003	0.040
380	580	0.005	0.003	-0.002	-0.004	0.031
380	590	0.005	0.003	-0.004	-0.005	0.026
380	600	0.004	0.003	-0.004	-0.004	0.019
380	610	0.004	0.003	-0.005	-0.002	0.013
380	620	0.003	0.003	-0.005	-0.002	0.008
380	630	0.003	0.003	-0.004	-0.002	0.003
380	640	0.003	0.003	-0.004	-0.001	-0.001
380	650	0.003	0.003	-0.003	-0.001	-0.005
380	660	0.002	0.003	-0.004	0.000	-0.004
380	670	0.002	0.003	-0.003	0.001	-0.006
380	680	0.002	0.003	-0.002	0.001	-0.007
380	690	0.002	0.003	-0.003	0.001	-0.007
390	420	-0.026	0.019	-0.054	-0.118	0.022

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
390	430	-0.016	0.001	-0.031	-0.069	-0.063
390	440	-0.017	0.009	-0.016	-0.094	-0.027
390	450	-0.008	0.004	0.029	-0.074	0.006
390	460	0.004	-0.006	0.047	-0.030	-0.025
390	470	0.006	-0.001	0.047	-0.029	-0.005
390	480	0.009	0.001	0.050	-0.022	0.017
390	490	0.012	-0.001	0.045	-0.005	0.029
390	500	0.012	0.000	0.034	0.000	0.040
390	510	0.012	0.001	0.026	-0.002	0.055
390	520	0.011	0.002	0.020	-0.004	0.059
390	530	0.010	0.003	0.015	-0.005	0.058
390	540	0.008	0.004	0.010	-0.009	0.053
390	550	0.007	0.005	0.006	-0.011	0.048
390	560	0.006	0.005	0.002	-0.012	0.043
390	570	0.005	0.005	0.000	-0.012	0.036
390	580	0.005	0.005	-0.002	-0.013	0.030
390	590	0.004	0.004	-0.003	-0.012	0.025
390	600	0.004	0.004	-0.004	-0.010	0.020
390	610	0.003	0.004	-0.005	-0.009	0.016
390	620	0.003	0.004	-0.004	-0.006	0.011
390	630	0.003	0.004	-0.004	-0.005	0.002
390	640	0.003	0.004	-0.003	-0.003	-0.001
390	650	0.003	0.004	-0.002	-0.003	-0.004
390	660	0.002	0.004	-0.003	-0.002	-0.004

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
390	670	0.002	0.003	-0.003	-0.001	-0.005
390	680	0.002	0.003	-0.002	0.000	-0.006
390	690	0.002	0.004	-0.003	-0.001	-0.004
400	430	-0.018	0.017	-0.038	-0.099	-0.028
400	440	-0.020	0.024	-0.024	-0.130	-0.003
400	450	-0.012	0.017	0.020	-0.112	0.018
400	460	0.000	0.008	0.038	-0.068	-0.011
400	470	0.003	0.011	0.038	-0.063	-0.001
400	480	0.006	0.011	0.042	-0.052	0.018
400	490	0.009	0.008	0.040	-0.033	0.026
400	500	0.010	0.008	0.030	-0.024	0.041
400	510	0.010	0.009	0.023	-0.024	0.054
400	520	0.009	0.008	0.017	-0.022	0.061
400	530	0.008	0.008	0.013	-0.022	0.058
400	540	0.007	0.008	0.009	-0.024	0.055
400	550	0.006	0.008	0.005	-0.024	0.054
400	560	0.005	0.008	0.002	-0.023	0.045
400	570	0.004	0.008	0.000	-0.021	0.039
400	580	0.004	0.007	-0.002	-0.021	0.035
400	590	0.003	0.006	-0.003	-0.019	0.029
400	600	0.003	0.006	-0.004	-0.016	0.024
400	610	0.002	0.005	-0.004	-0.013	0.019
400	620	0.003	0.005	-0.004	-0.012	0.012
400	630	0.002	0.005	-0.002	-0.009	0.007

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
400	640	0.003	0.004	-0.002	-0.008	0.002
400	650	0.003	0.005	-0.002	-0.005	-0.002
400	660	0.002	0.004	-0.001	-0.004	-0.003
400	670	0.002	0.004	-0.002	-0.002	-0.004
400	680	0.002	0.004	-0.002	-0.002	-0.005
400	690	0.002	0.004	-0.003	-0.001	-0.005
410	440	-0.020	0.035	-0.003	-0.141	0.070
410	450	-0.013	0.029	0.031	-0.129	0.039
410	460	-0.002	0.019	0.050	-0.087	0.006
410	470	0.001	0.022	0.047	-0.082	0.000
410	480	0.005	0.021	0.047	-0.068	-0.004
410	490	0.008	0.017	0.042	-0.046	0.003
410	500	0.009	0.016	0.033	-0.037	0.019
410	510	0.009	0.016	0.025	-0.034	0.033
410	520	0.008	0.015	0.019	-0.031	0.040
410	530	0.007	0.014	0.013	-0.030	0.043
410	540	0.006	0.013	0.009	-0.031	0.043
410	550	0.005	0.012	0.005	-0.029	0.041
410	560	0.005	0.012	0.003	-0.029	0.038
410	570	0.004	0.010	0.001	-0.027	0.030
410	580	0.003	0.009	-0.001	-0.025	0.029
410	590	0.003	0.008	-0.002	-0.022	0.022
410	600	0.002	0.007	-0.003	-0.019	0.020
410	610	0.002	0.006	-0.003	-0.017	0.015

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
410	620	0.002	0.006	-0.003	-0.014	0.011
410	630	0.002	0.005	-0.003	-0.011	0.004
410	640	0.002	0.005	-0.002	-0.010	0.001
410	650	0.002	0.005	-0.002	-0.007	-0.001
410	660	0.002	0.004	-0.002	-0.006	-0.004
410	670	0.002	0.004	-0.002	-0.004	-0.004
410	680	0.002	0.004	-0.002	-0.002	-0.005
410	690	0.002	0.004	-0.002	-0.003	-0.005
420	450	0.001	0.013	0.052	-0.056	-0.036
420	460	0.008	0.013	0.060	-0.043	-0.063
420	470	0.011	0.017	0.058	-0.039	-0.074
420	480	0.013	0.019	0.051	-0.035	-0.073
420	490	0.015	0.020	0.041	-0.026	-0.056
420	500	0.015	0.021	0.029	-0.020	-0.031
420	510	0.014	0.021	0.020	-0.019	-0.012
420	520	0.012	0.021	0.012	-0.021	0.007
420	530	0.011	0.020	0.008	-0.025	0.015
420	540	0.009	0.019	0.003	-0.027	0.021
420	550	0.008	0.018	0.000	-0.028	0.023
420	560	0.007	0.017	-0.003	-0.028	0.022
420	570	0.006	0.015	-0.004	-0.028	0.020
420	580	0.005	0.013	-0.005	-0.026	0.018
420	590	0.004	0.012	-0.006	-0.022	0.017
420	600	0.003	0.010	-0.007	-0.021	0.015

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
420	610	0.003	0.009	-0.006	-0.018	0.012
420	620	0.003	0.008	-0.006	-0.016	0.009
420	630	0.002	0.007	-0.005	-0.012	0.005
420	640	0.003	0.006	-0.003	-0.010	0.001
420	650	0.002	0.006	-0.003	-0.007	0.000
420	660	0.002	0.005	0.003	-0.006	-0.002
420	670	0.002	0.005	-0.003	-0.005	-0.003
420	680	0.002	0.004	-0.003	-0.004	-0.003
420	690	0.001	0.004	-0.003	-0.002	-0.005
430	460	0.013	0.010	0.050	-0.012	-0.121
430	470	0.017	0.017	0.046	-0.010	-0.143
430	480	0.019	0.022	0.038	-0.006	-0.136
430	490	0.020	0.025	0.025	-0.001	-0.115
430	500	0.020	0.027	0.012	0.003	-0.089
430	510	0.019	0.028	0.004	0.001	-0.064
430	520	0.018	0.028	-0.003	-0.004	-0.040
430	530	0.015	0.027	-0.008	-0.009	-0.024
430	540	0.013	0.027	-0.011	-0.014	-0.016
430	550	0.012	0.025	-0.014	-0.016	-0.004
430	560	0.010	0.023	-0.016	-0.018	-0.003
430	570	0.009	0.021	-0.017	-0.017	-0.003
430	580	0.007	0.018	-0.016	-0.018	0.002
430	590	0.006	0.016	-0.016	-0.016	0.002
430	600	0.005	0.014	-0.015	-0.015	0.003

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
430	610	0.005	0.013	-0.015	-0.012	0.003
430	620	0.004	0.011	-0.013	-0.011	0.001
430	630	0.004	0.010	-0.012	-0.007	0.000
430	640	0.003	0.009	-0.009	-0.006	-0.003
430	650	0.003	0.007	-0.009	-0.004	-0.004
430	660	0.003	0.007	-0.008	-0.002	-0.006
430	670	0.003	0.006	-0.007	-0.002	-0.005
430	680	0.002	0.005	-0.006	0.001	-0.006
430	690	0.002	0.005	-0.006	0.000	-0.006
440	470	0.014	0.018	0.025	0.000	-0.127
440	480	0.018	0.023	0.016	0.006	-0.131
440	490	0.020	0.027	0.005	0.013	-0.116
440	500	0.020	0.030	-0.005	0.018	-0.094
404	510	0.020	0.032	-0.012	0.017	-0.079
440	520	0.019	0.032	-0.019	0.013	-0.056
440	530	0.017	0.032	-0.022	0.006	-0.040
440	540	0.015	0.031	-0.023	0.000	-0.029
440	550	0.014	0.029	-0.024	-0.002	-0.022
440	560	0.012	0.027	-0.026	-0.006	-0.017
440	570	0.011	0.024	-0.024	-0.007	-0.016
440	580	0.009	0.022	-0.024	-0.006	-0.012
440	590	0.008	0.019	-0.022	-0.005	-0.010
440	600	0.007	0.017	-0.022	-0.003	-0.004
440	610	0.006	0.015	-0.020	0.003	-0.004

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
440	620	0.005	0.013	-0.017	-0.001	-0.004
440	630	0.005	0.012	-0.017	0.000	-0.003
440	640	0.004	0.010	-0.014	0.002	-0.003
440	650	0.004	0.008	-0.013	0.003	-0.003
440	660	0.003	0.008	-0.011	0.004	-0.002
440	670	0.003	0.007	-0.010	0.004	-0.004
440	680	0.003	0.007	-0.010	0.005	-0.002
440	690	0.002	0.006	-0.009	0.005	-0.002
450	480	0.013	0.021	-0.001	0.009	-0.082
450	490	0.016	0.026	-0.010	0.017	-0.079
450	500	0.018	0.030	-0.019	0.024	-0.067
450	510	0.019	0.033	-0.025	0.025	-0.055
450	520	0.018	0.035	-0.028	0.020	-0.045
450	530	0.017	0.035	-0.029	0.013	-0.035
450	540	0.015	0.034	-0.031	0.007	-0.024
450	550	0.014	0.033	-0.032	0.003	-0.019
450	560	0.013	0.031	-0.031	0.002	-0.017
450	570	0.011	0.028	-0.030	0.001	-0.015
450	580	0.010	0.025	-0.029	0.000	-0.012
450	590	0.009	0.022	-0.028	0.001	-0.009
450	600	0.008	0.020	-0.026	0.002	-0.007
450	610	0.007	0.017	-0.024	0.003	-0.006
450	620	0.006	0.015	-0.022	0.004	-0.004
450	630	0.005	0.013	-0.021	0.005	-0.001

ex (nm)	em (nm)	evec3	evec5	evec10	evec16	evec18
450	640	0.004	0.011	-0.018	0.006	-0.003
450	650	0.004	0.010	0.016	0.005	-0.003
450	660	0.003	0.009	-0.014	0.006	-0.004
450	670	0.003	0.008	-0.012	0.007	-0.003
450	680	0.003	0.007	-0.011	0.007	-0.004
450	690	0.002	0.006	-0.010	0.006	-0.003
460	490	0.013	0.023	-0.024	0.024	-0.050
460	500	0.015	0.028	-0.033	0.032	-0.049
460	510	0.017	0.033	-0.039	0.035	-0.044
460	520	0.017	0.035	-0.042	0.030	-0.039
460	530	0.016	0.037	-0.044	0.022	-0.034
460	540	0.016	0.037	-0.045	0.017	-0.027
460	550	0.015	0.035	-0.045	0.014	-0.025
460	560	0.014	0.034	-0.044	0.012	-0.022
460	570	0.012	0.031	-0.043	0.011	-0.023
460	580	0.011	0.028	-0.042	0.010	-0.021
460	590	0.010	0.025	-0.039	0.011	-0.018
460	600	0.009	0.022	-0.037	0.012	-0.013
460	610	0.008	0.020	-0.035	0.013	-0.011
460	620	0.007	0.017	-0.031	0.013	-0.006
460	630	0.006	0.015	-0.028	0.014	-0.008
460	640	0.005	0.013	-0.025	0.013	-0.007
460	650	0.005	0.011	-0.022	0.012	-0.006
460	660	0.004	0.010	-0.020	0.012	-0.003

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
460	670	0.004	0.009	-0.018	0.011	-0.008
460	680	0.003	0.008	-0.016	0.011	-0.004
460	690	0.002	0.007	-0.012	0.009	0.004
470	500	0.011	0.024	-0.029	0.025	-0.007
470	510	0.012	0.030	-0.034	0.025	-0.005
470	520	0.013	0.035	-0.039	0.021	-0.002
470	530	0.013	0.037	-0.040	0.013	0.003
470	540	0.013	0.038	-0.042	0.008	0.003
470	550	0.012	0.038	-0.042	0.006	0.002
470	560	0.012	0.036	-0.043	0.006	0.001
470	570	0.011	0.034	-0.043	0.006	-0.002
470	580	0.010	0.031	-0.041	0.007	-0.001
470	590	0.009	0.028	-0.040	0.009	-0.002
470	600	0.009	0.025	-0.038	0.011	0.001
470	610	0.008	0.023	-0.035	0.014	0.002
470	620	0.007	0.020	-0.033	0.014	0.002
470	630	0.006	0.017	-0.029	0.015	0.003
470	640	0.005	0.015	-0.027	0.016	0.001
470	650	0.005	0.013	-0.024	0.016	-0.001
470	660	0.004	0.011	-0.021	0.015	0.003
470	670	0.004	0.010	-0.019	0.013	0.000
470	680	0.003	0.009	-0.016	0.012	0.000
470	690	0.003	0.007	-0.014	0.012	-0.002
480	510	0.009	0.024	-0.031	0.024	-0.003

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
480	520	0.010	0.031	-0.037	0.017	-0.005
480	530	0.011	0.036	-0.040	0.011	-0.007
480	540	0.011	0.038	-0.043	0.005	-0.009
480	550	0.011	0.038	-0.046	0.006	-0.010
480	560	0.011	0.038	-0.047	0.008	-0.013
480	570	0.011	0.036	-0.047	0.008	-0.014
480	580	0.010	0.033	-0.046	0.010	-0.016
480	590	0.010	0.030	-0.046	0.014	-0.011
480	600	0.009	0.028	-0.043	0.018	-0.011
480	610	0.008	0.025	-0.041	0.020	-0.011
480	620	0.008	0.022	-0.038	0.022	-0.009
480	630	0.007	0.019	-0.034	0.022	-0.007
480	640	0.006	0.017	-0.031	0.022	-0.006
480	650	0.005	0.015	-0.028	0.020	-0.005
480	660	0.005	0.012	-0.025	0.020	-0.005
480	670	0.004	0.011	-0.022	0.018	-0.003
480	680	0.004	0.010	-0.018	0.015	-0.004
480	690	0.003	0.009	-0.017	0.015	-0.003
490	520	0.006	0.026	-0.031	0.008	0.010
490	530	0.007	0.033	-0.036	-0.001	0.010
490	540	0.008	0.037	-0.040	-0.005	0.005
490	550	0.008	0.039	-0.045	-0.003	0.004
490	560	0.009	0.038	-0.048	0.001	0.000
490	570	0.009	0.037	-0.050	0.004	-0.006

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
490	580	0.009	0.034	-0.049	0.009	-0.006
490	590	0.009	0.032	-0.048	0.012	-0.006
490	600	0.008	0.029	-0.047	0.017	-0.007
490	610	0.008	0.026	-0.046	0.022	-0.004
490	620	0.007	0.023	-0.042	0.023	-0.004
490	630	0.007	0.021	-0.039	0.024	-0.003
490	640	0.006	0.018	-0.035	0.025	-0.003
490	650	0.006	0.016	-0.032	0.024	-0.003
490	660	0.005	0.014	-0.029	0.023	-0.001
490	670	0.004	0.012	-0.027	0.023	-0.001
490	680	0.004	0.011	-0.021	0.019	-0.002
490	690	0.004	0.010	-0.021	0.019	-0.001
500	530	0.002	0.030	-0.024	-0.017	0.026
500	540	0.004	0.035	-0.029	-0.022	0.026
500	550	0.005	0.038	-0.035	-0.020	0.021
500	560	0.006	0.038	-0.041	-0.014	0.015
500	570	0.007	0.038	-0.044	-0.010	0.012
500	580	0.007	0.035	-0.044	-0.003	0.009
500	590	0.007	0.033	-0.044	0.004	0.008
500	600	0.007	0.030	-0.044	0.010	0.007
500	610	0.007	0.028	-0.043	0.014	0.010
500	620	0.006	0.025	-0.041	0.020	0.007
500	630	0.006	0.022	-0.040	0.021	0.007
500	640	0.006	0.020	-0.036	0.022	0.007

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
500	650	0.005	0.017	-0.033	0.025	0.006
500	660	0.005	0.016	-0.030	0.022	0.007
500	670	0.004	0.014	-0.027	0.020	0.003
500	680	0.004	0.011	-0.024	0.021	0.000
500	690	0.004	0.011	-0.022	0.020	0.002
510	540	0.000	0.032	-0.022	-0.036	0.036
510	550	0.002	0.036	-0.029	-0.032	0.034
510	560	0.003	0.037	-0.036	-0.027	0.026
510	570	0.005	0.037	-0.042	-0.018	0.022
510	580	0.005	0.035	-0.044	-0.010	0.017
510	590	0.005	0.033	-0.043	-0.003	0.010
510	600	0.006	0.030	-0.043	0.005	0.013
510	610	0.006	0.028	-0.043	0.012	0.008
510	620	0.006	0.025	-0.041	0.017	0.011
510	630	0.006	0.023	-0.038	0.019	0.008
510	640	0.006	0.020	-0.037	0.023	0.008
510	650	0.005	0.018	-0.034	0.025	0.006
510	660	0.005	0.016	-0.029	0.022	0.001
510	670	0.004	0.014	-0.028	0.022	0.005
510	680	0.004	0.013	-0.024	0.020	0.005
510	690	0.003	0.012	-0.021	0.019	0.006
520	550	-0.001	0.033	-0.024	-0.040	0.042
520	560	0.001	0.035	-0.032	-0.031	0.033
520	570	0.003	0.037	-0.039	-0.024	0.027

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
520	580	0.004	0.035	-0.041	-0.017	0.019
520	590	0.004	0.033	-0.041	-0.009	0.017
520	600	0.005	0.030	-0.042	-0.004	0.013
520	610	0.005	0.028	-0.041	0.005	0.012
520	620	0.005	0.026	-0.040	0.011	0.011
520	630	0.005	0.023	-0.038	0.016	0.007
520	640	0.005	0.021	-0.037	0.018	0.007
520	650	0.005	0.019	-0.034	0.023	0.006
520	660	0.005	0.017	-0.032	0.021	0.005
520	670	0.004	0.015	-0.029	0.021	0.003
520	680	0.004	0.014	-0.028	0.021	0.004
520	690	0.004	0.013	-0.025	0.017	0.005
530	560	0.000	0.032	-0.030	-0.032	0.040
530	570	0.002	0.033	-0.035	-0.024	0.030
530	580	0.003	0.032	-0.037	-0.018	0.022
530	590	0.003	0.030	-0.037	-0.013	0.015
530	600	0.004	0.029	-0.038	-0.004	0.012
530	610	0.004	0.027	-0.038	0.003	0.010
530	620	0.005	0.025	-0.038	0.011	0.009
530	630	0.005	0.023	-0.037	0.015	0.011
530	640	0.005	0.021	-0.034	0.019	0.005
530	650	0.005	0.019	-0.033	0.022	0.003
530	660	0.004	0.017	-0.031	0.020	0.003
530	670	0.004	0.015	-0.026	0.021	0.000

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
530	680	0.004	0.013	-0.024	0.023	-0.001
530	690	0.004	0.015	-0.025	0.021	0.007
540	570	0.003	0.029	-0.038	-0.012	0.031
540	580	0.003	0.028	-0.038	-0.009	0.019
540	590	0.004	0.027	-0.037	-0.007	0.015
540	600	0.004	0.026	-0.036	0.000	0.011
540	610	0.004	0.025	-0.038	0.006	0.010
540	620	0.005	0.024	-0.036	0.012	0.007
540	630	0.005	0.022	-0.035	0.015	0.005
540	640	0.005	0.020	-0.033	0.020	0.006
540	650	0.005	0.018	-0.031	0.021	-0.001
540	660	0.005	0.017	-0.031	0.022	0.004
540	670	0.004	0.015	-0.027	0.024	0.001
540	680	0.004	0.015	-0.025	0.023	0.001
540	690	0.004	0.013	-0.025	0.021	0.004
550	580	0.005	0.025	-0.043	0.005	0.021
550	590	0.004	0.023	-0.038	0.005	0.014
550	600	0.005	0.024	-0.036	0.010	0.013
550	610	0.005	0.023	-0.037	0.011	0.011
550	620	0.005	0.022	-0.037	0.018	0.011
550	630	0.005	0.021	-0.036	0.020	0.008
550	640	0.005	0.019	-0.034	0.024	0.005
550	650	0.005	0.018	-0.032	0.025	0.006
550	660	0.005	0.017	-0.031	0.025	0.005

ex(nm)	em(nm)	evec3	evec5	evec10	evec16	evec18
550	670	0.005	0.015	-0.027	0.025	0.002
550	680	0.004	0.015	-0.025	0.025	0.004
550	690	0.004	0.015	-0.025	0.024	0.006

APPENDIX III: REFERENCES

The disclosures of the following publications, patents and applications are expressly incorporated herein by reference, as are any of the other references mentioned above:

A two-stage fluorescence diagnostic method is disclosed in detail in application Serial No. 08/060,432, filed May 12, 1993, and is assigned to the same assignee as the present invention.

An application entitled "Optical Method And apparatus for the Diagnosis of Cervical Precancers using Raman and Fluorescence Spectroscopies" (Richards-Kortum *et al.*) was filed on March 14, 1995, and is assigned to the same assignee as the present invention.

Lui *et al.*, "Fluorescence and Time-Resolved Light Scattering as Optical Diagnostic Techniques to Separate Diseased and Normal Biomedical Media," *J. Photochem. Photobiol. B: Biol.* 16, 187-209, 1992.

Wong *et al.*, "Infrared Spectroscopy of Human Cervical Cells: Evidence of Extensive Structural Changes during Carcinogenesis", *Proc. Nat'l Acad. Sci. USA*, 88, 10988-10992, 1991.

Kurman RJ, Henson DE, Herbst AL, Noller KL, Shiffman MH, "Interim guidelines for management of abnormal cervical cytology," *JAMA*, 271, 1866-1869, 1994.

Davison JM, Marty JJ, "Detecting premalignant cervical lesions: Contributions of screening colposcopy to cytology," *J Reproductive Medicine*, 39, 388-392, 1994.

Banda-Gamboa, Hugo, et al, "Automation in cervical cytology: an overview," *Anal Cell Path*, 4 (1992) 25-48.

Chance B, Thorell L, "Localization and kinetics of reduced PN in living cells by microfluorimetry," *J Biol Chem* 234:3044, 1959.

Caspersson TC, *Cell Structure and Function*, ch 4, Norton & Co, New York, 1950.

Aubin JE, "Autofluorescence of Viable Cultured Mammalian Cells," *J Histochem Cytochem*, 27(1), 36-43, 1979.

Benson RC, et al, "Cellular Autofluorescence: Is It Due to Flavins?" *J Histochem Cytochem*, 27(1), 44-48, 1979.

Tamura M, ... Chance B, "In Vivo Study of Tissue Oxygen ...," *Annu Rev Physiol*, 1989, 51:813-814.

Hutchinson, ML, Agarwal, P, Denault, T, Berger, B, Cibas, ES, "A New Look at Cervical Cytology," *Acta Cytologica*, 36, 4:499-504, 1992.

Taylor DG, Demas JN, "Light intensity measurements I: Large area bolometers with uW sensitivities and absolute calibration of the rhodamine B quantum counter," *Anal Chem*, 51, 712-717, 1979.

Vooijs GP, "Benign proliferative reactions, intraepithelial neoplasia and invasive cancer of the uterine cervix," in Bibbo M (ed), *Comprehensive Cytopathology*, WB Saunders Company, pp 176-177, 1991.

Dillon WR and Goldstein M, "Multivariate Analysis Methods and Applications," Chapter 10. Wiley & Sons, New York (1985)

CLAIMS:

1. A method of detecting tissue abnormality in a tissue sample *in vitro* comprising:

(i) providing a tissue sample;

(ii) sequentially illuminating said tissue sample *in vitro* with a set of at least two electromagnetic radiation wavelengths selected to cause said tissue sample to produce a set of fluorescence intensity spectra indicative of tissue abnormality;

(iii) detecting said set of fluorescence intensity spectra emitted from said tissue sample as a result of illumination with each of said wavelengths; and

(iv) calculating from said set of fluorescence intensity spectra, a probability that said tissue sample is normal or abnormal.

2. The method of claim 1, wherein said calculating step comprises, conducting principal component analysis of said fluorescent spectra relative to a set of preprocessed spectra obtained from tissue samples of known pathology.

3. The method of claim 2, wherein said principal component analysis does not include the highest and lowest order principal components.

4. The method of claim 3, wherein said principal component analysis comprises a Fisher's determinant analysis.

5. The method of claim 1, wherein said calculating step comprises, normalizing said spectra relative to a maximum intensity within said spectra.

6. The method of claim 5, wherein said calculating step further comprises, mean-scaling said spectra as a function of a mean intensity of spectra.

7. The method of claim 1, wherein said providing step comprises obtaining said tissue sample by biopsy.

8. The method of claim 7, wherein said providing step further comprises ethanol fixation of said tissue sample.

9. The method of claim 8, wherein said providing step even further comprises generating a monolayer cell touch preparation or a pellet.

10. The method of claim 1, wherein said set of at least two electromagnetic wavelengths are 250 nm, 550 nm and all wavelengths between 250 nm and 550 nm at 10 nm intervals.

11. The method of claim 1, wherein said detecting step comprises, detecting an intensity of fluorescence at 250

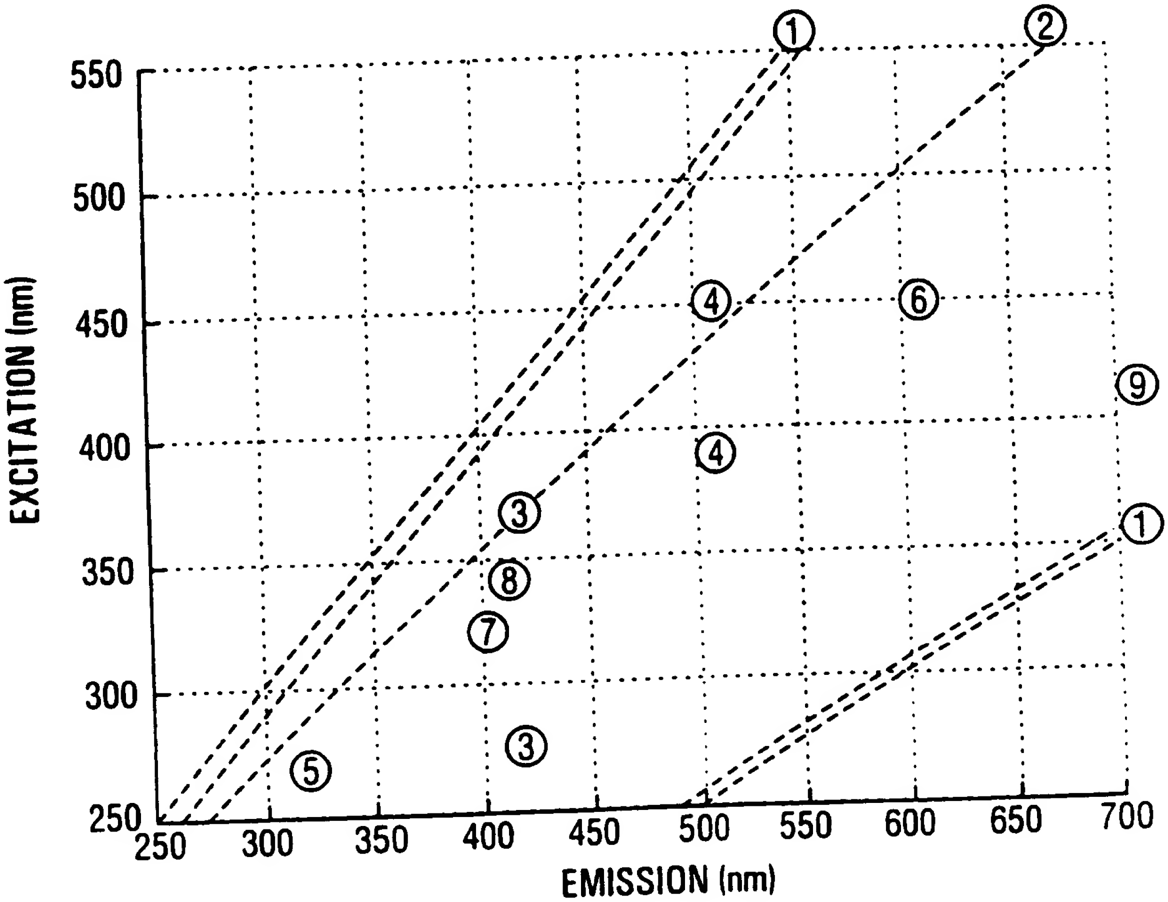
nm, 700 nm and all wavelengths between 250 nm and 700 nm at 10 nm intervals.

12. The method of claim 1, wherein said illuminating comprises, illuminating said sample substantially normal to a surface of said sample, and wherein said detecting step comprises, detecting said spectra at an angle of approximately 20° from normal.

13. The method of claim 1, wherein at least 10 sequential electromagnetic radiation wavelengths are used in said illuminating step to produce a set of at least 10 different fluorescence intensity spectra in said detecting step, each spectra comprising fluorescence intensity at at least 11 wavelengths.

14. The method of claim 13, wherein at least 30 sequential electromagnetic radiation wavelengths are used in said illuminating step to produce a set of at least 30 different fluorescence intensity spectra in said detecting step, each spectra comprising fluorescence intensity at at least 31 wavelengths.

15. The method of claim 14, wherein at least 50 sequential electromagnetic radiation wavelengths are used in said illuminating step to produce a set of at least 50 different fluorescence intensity spectra in said detecting step, each said spectra comprising fluorescence intensity at at least 51 wavelengths.



550
500
450
400
350
300
250
EXCITATION (nm)
250
300
350
400
450
500
550
600
650
700
EMISSION (nm)
1
2
3
4
5
6
7
8
9

FIG. 1

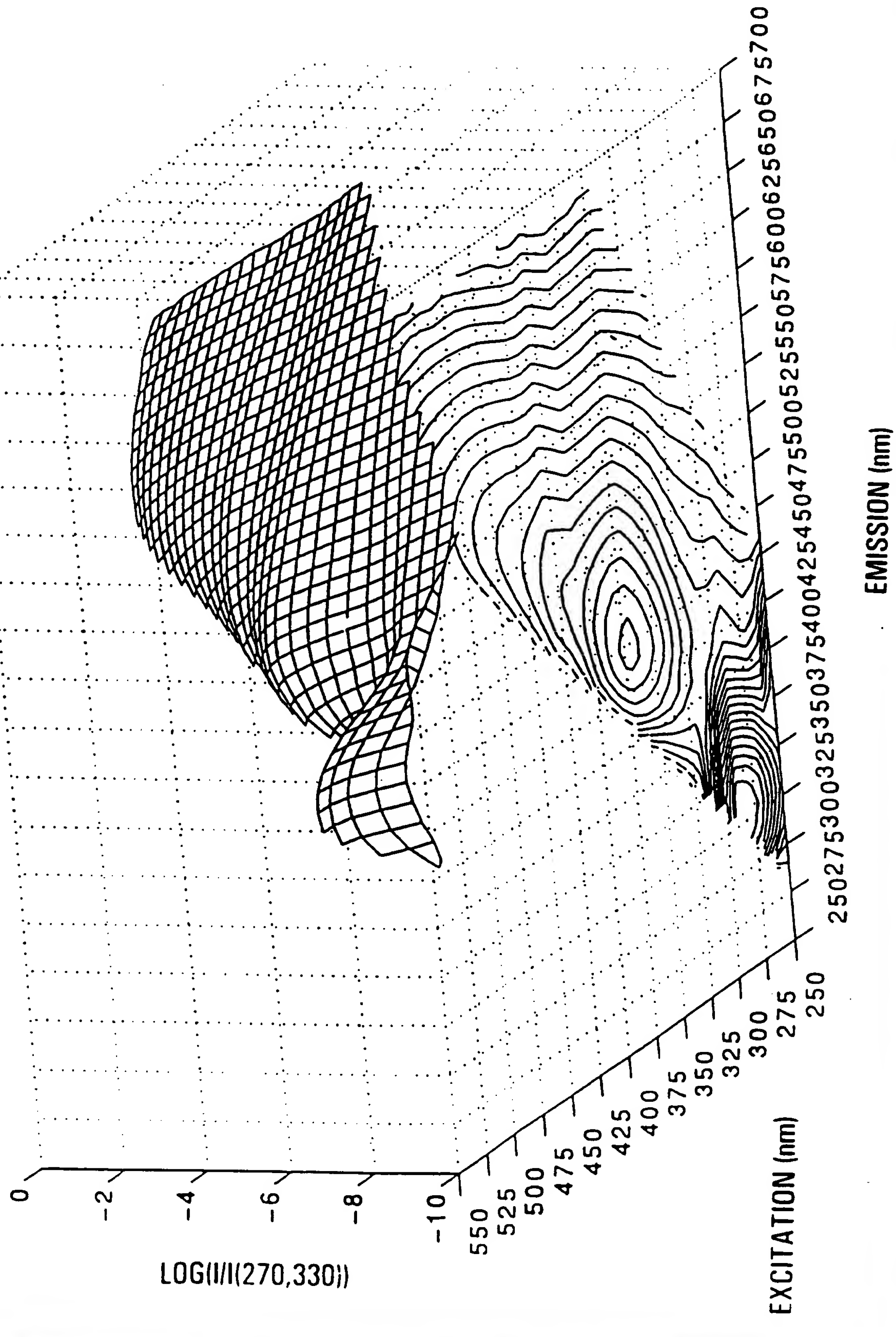

EMISSION (nm)
250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700
EXCITATION (nm)
250 275 300 325 350 375 400 425 450 475 500 525 550
LOG(I/I(270,330))
0 −2 −4 −6 −8 −10

FIG. 2

FIG. 3

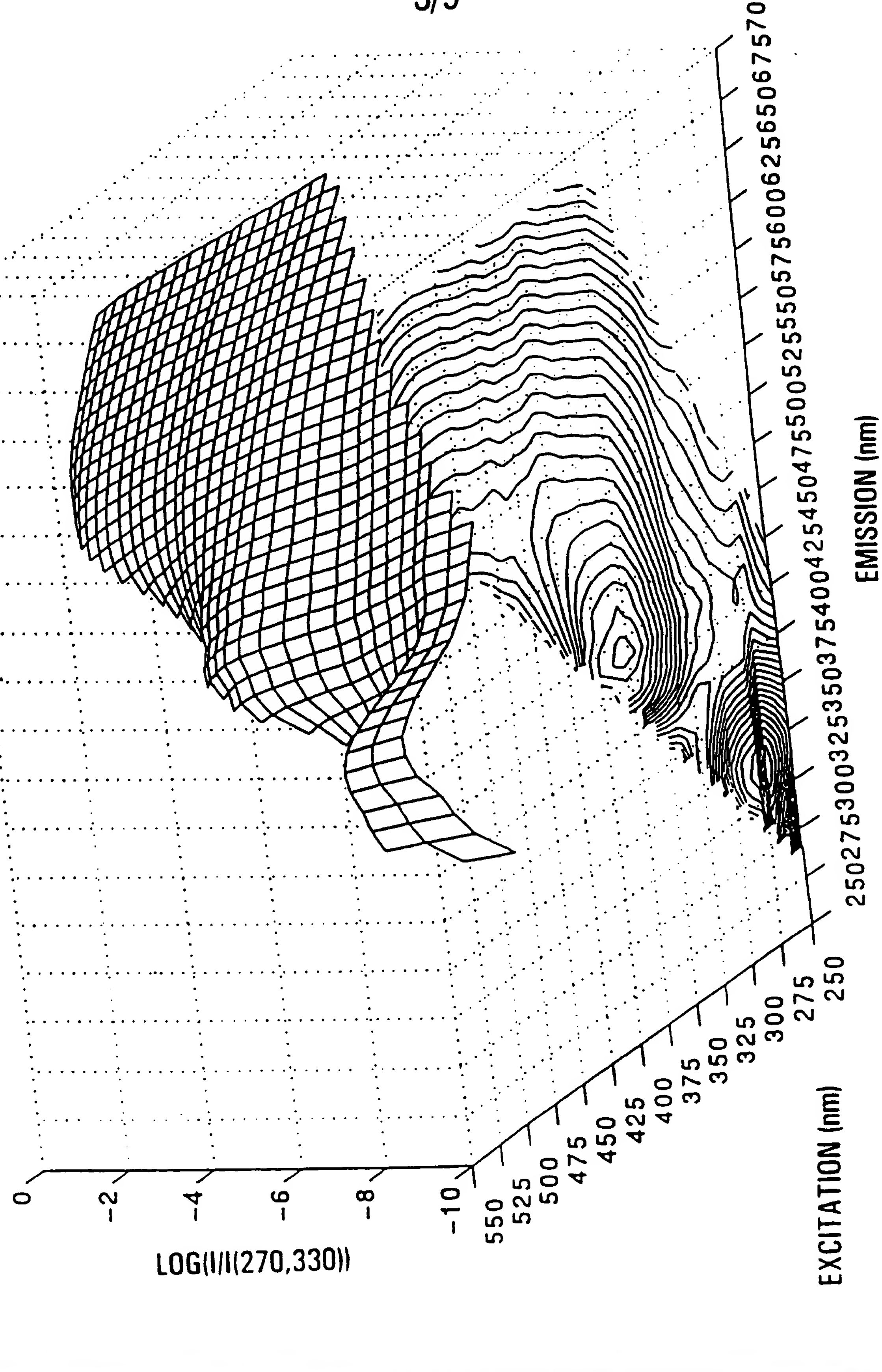
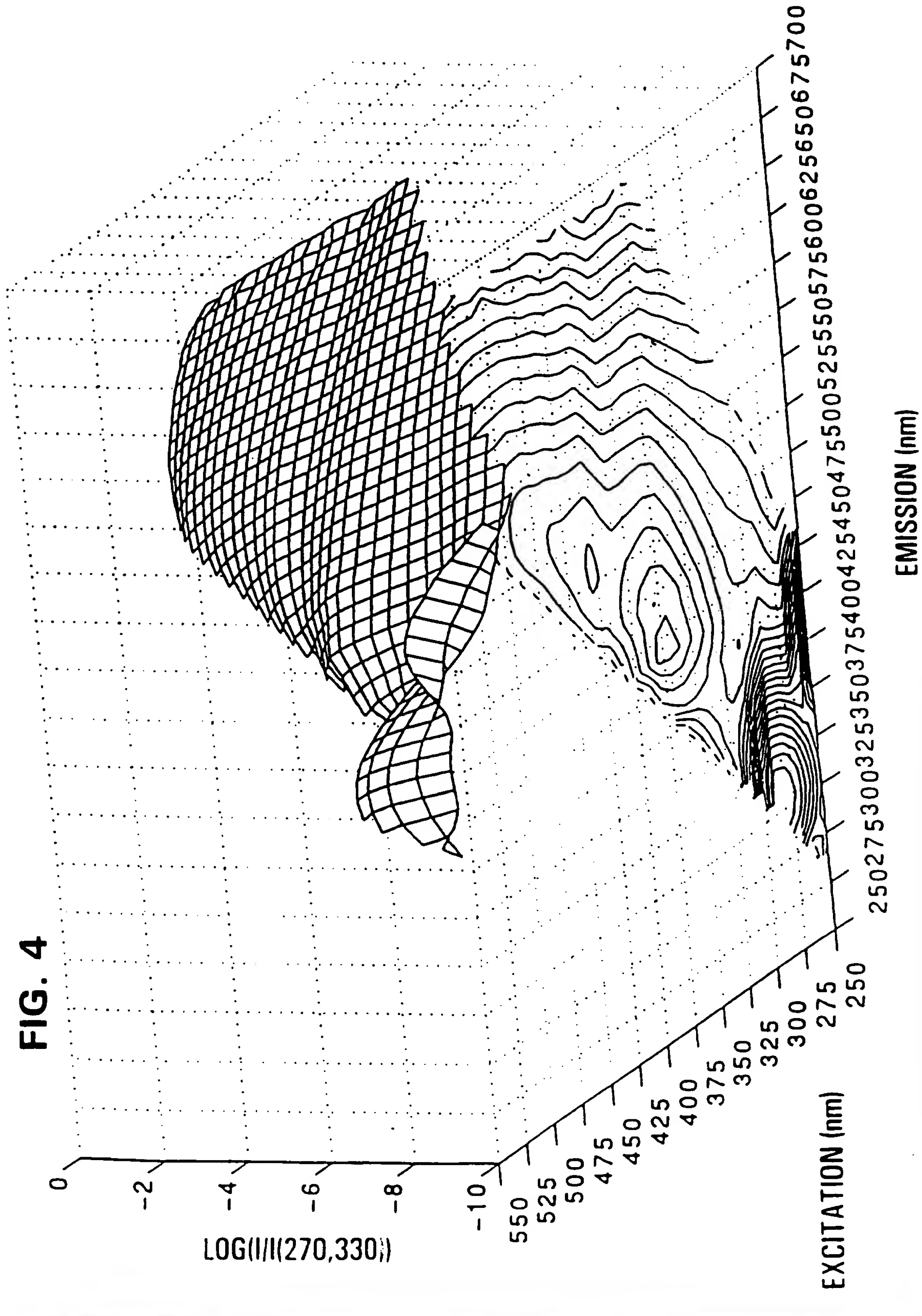

LOG(I/I(270,330))
0
-2
-4
-6
-8
-10
EXCITATION (nm)
550 525 500 475 450 425 400 375 350 325 300 275 250
EMISSION (nm)
250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700

FIG. 4


LOG(I/I(270,330))
0
-2
-4
-6
-8
-10
EXCITATION (nm)
550
525
500
475
450
425
400
375
350
325
300
275
250
EMISSION (nm)
250
275
300
325
350
375
400
425
450
475
500
525
550
575
600
625
650
675
700

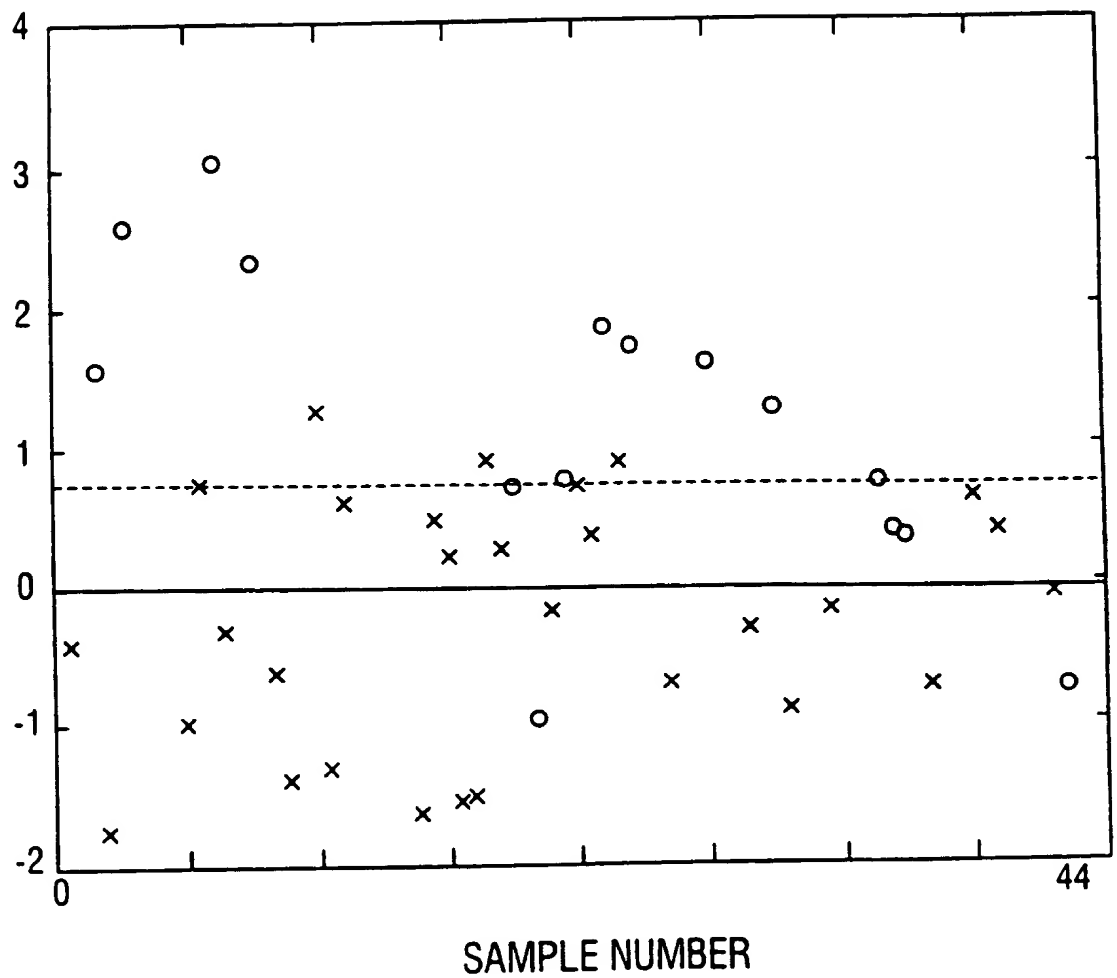


FIG. 5

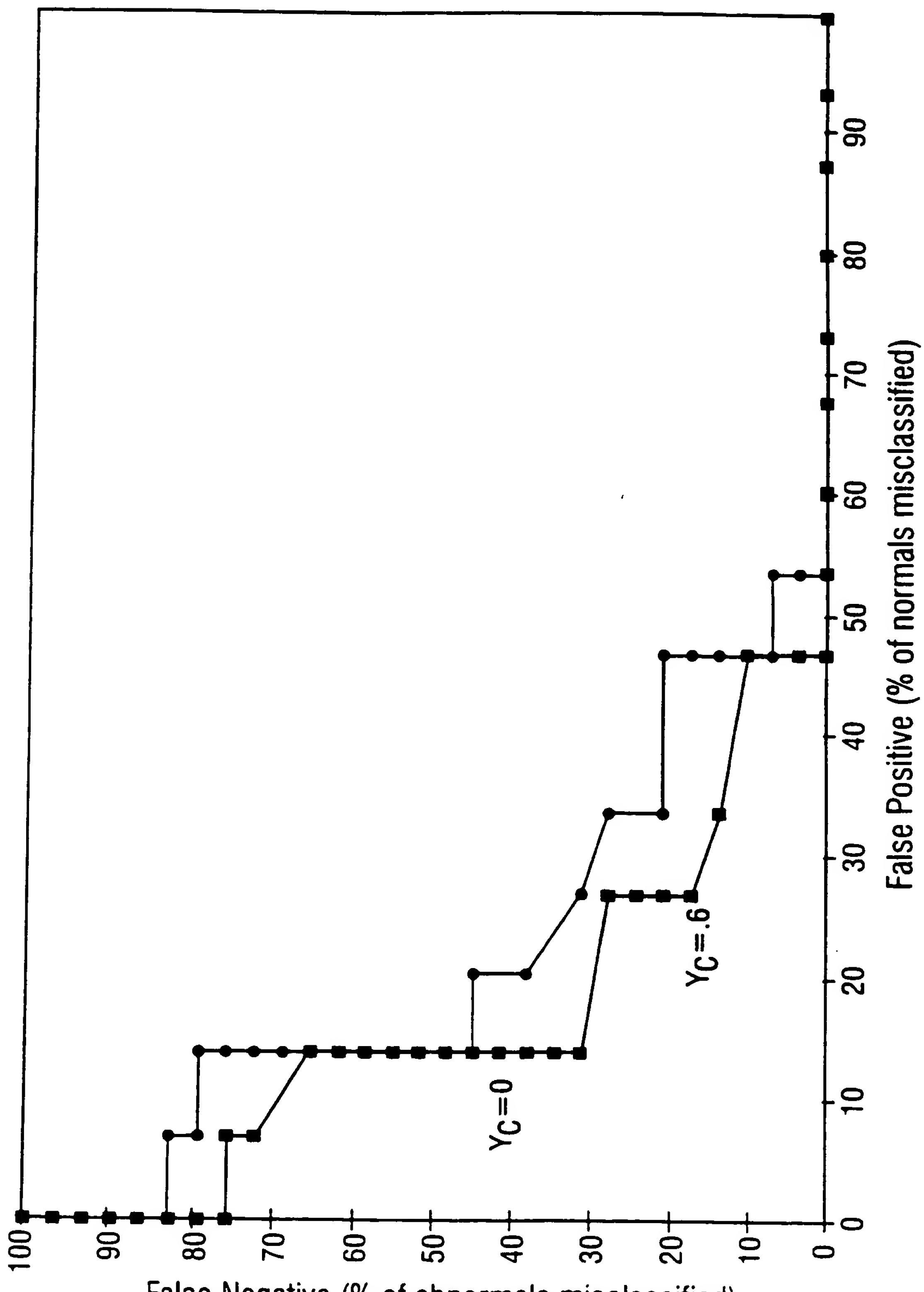

False Negative (% of abnormals misclassified)
False Positive (% of normals misclassified)
100
90
80
70
60
50
40
30
20
10
0
YC=0
YC=.6

FIG. 6

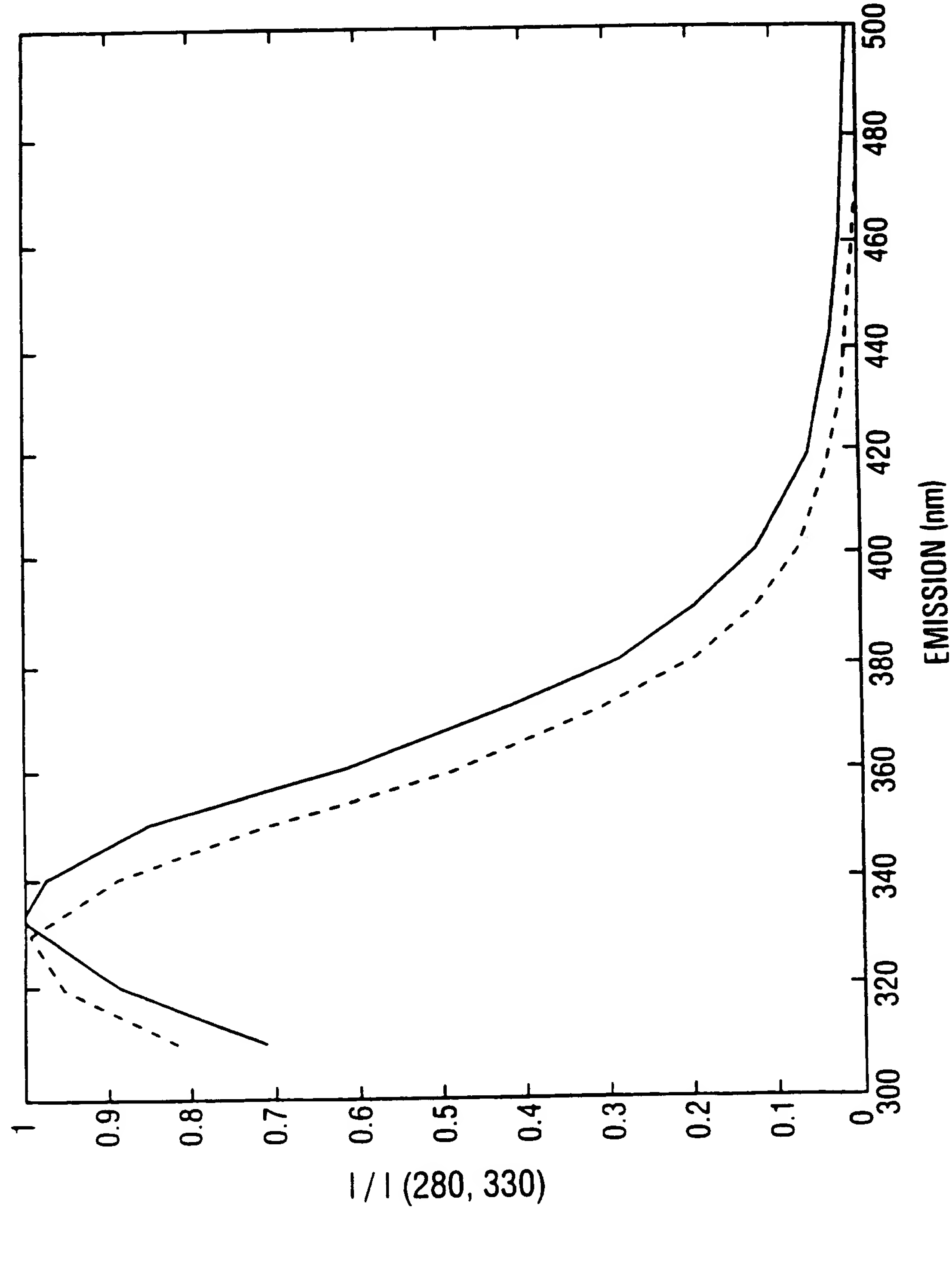

I / I (280, 330)
EMISSION (nm)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
300
320
340
360
380
400
420
440
460
480
500

FIG. 7

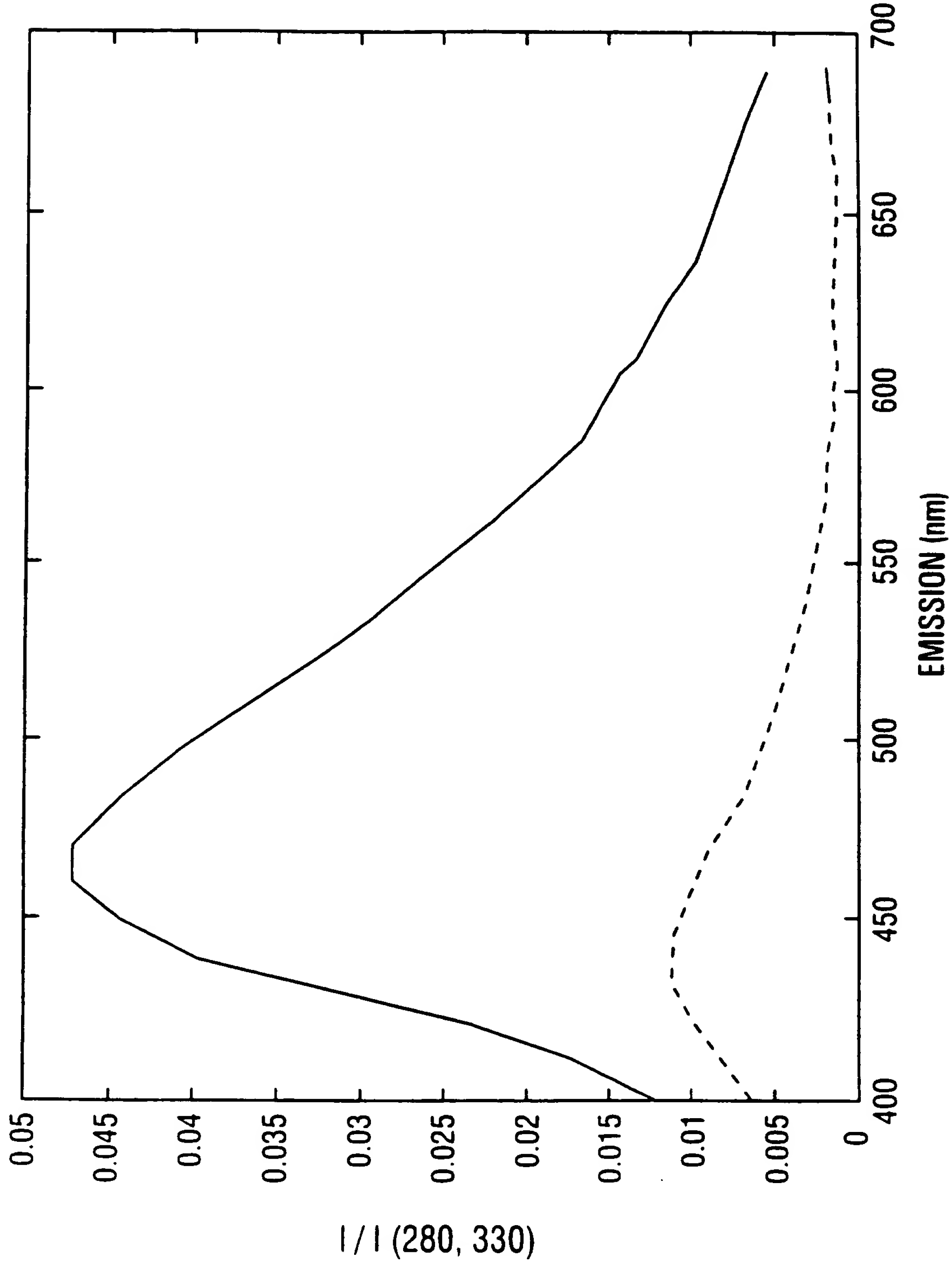

I / I (280, 330)
0.05
0.045
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0
EMISSION (nm)
400
450
500
550
600
650
700

FIG. 8

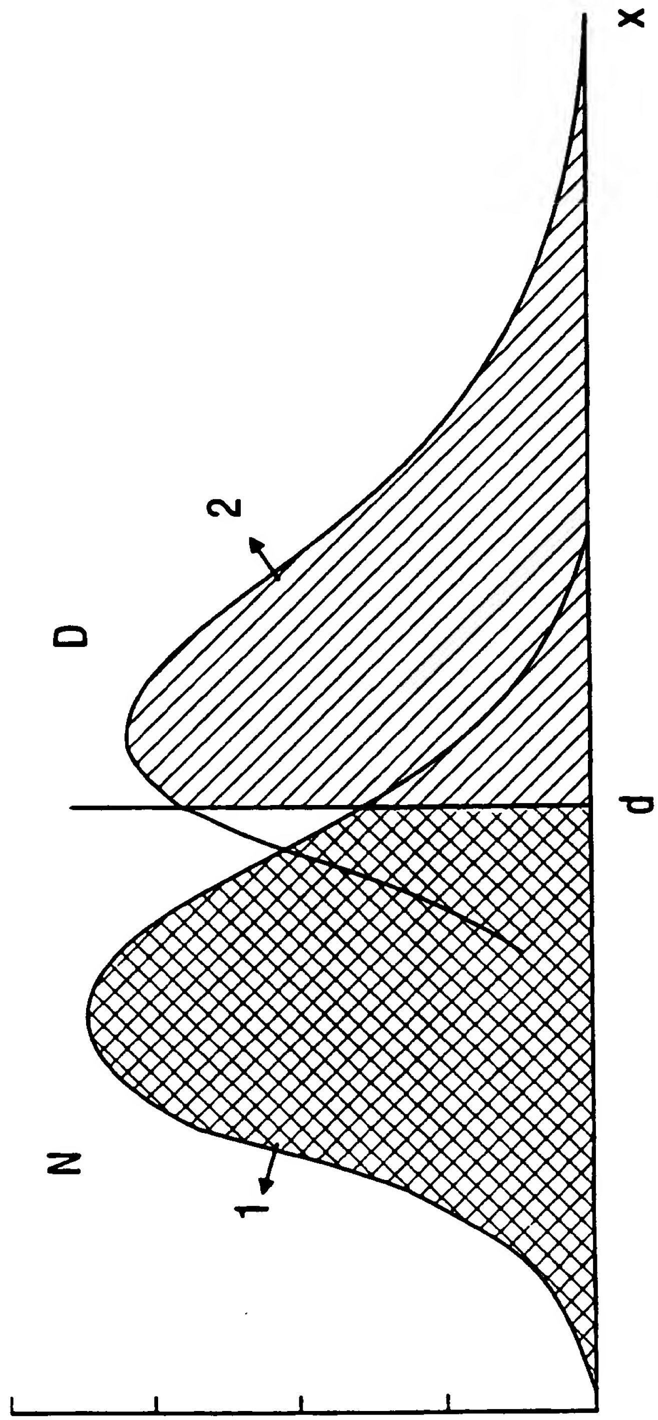


FIG. 9

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/US 96/04305

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N21/64

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO,A,90 12536 (MIT) 1 November 1990 see abstract see page 13, line 10 - line 18 see page 22, line 22 - page 23, line 2 see page 28, line 19 - line 25	1
A	see figures 32,33	10,11, 13-15

X	WO,A,90 06718 (MIT) 28 June 1990 see abstract	1
Y	see claims 1,4,5; figure 3	2,3

Y	US,A,5 348 003 (CARO) 20 September 1994 see column 1, paragraph 1 see column 2, line 4 - line 15 see column 14, line 3 - line 23	2,3

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

Date of the actual completion of the international search	Date of mailing of the international search report
4 July 1996	12.07.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Thomas, R.M.

INTERNATIONAL SEARCH REPORT

Inter   nal Application No
PCT/US 96/04305

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO,A,94 26168 (UNIVERSITY OF TEXAS) 24 November 1994 see page 2, line 30 - line 35 see page 5, line 35 - page 6, line 20 see page 17, line 3 - line 6 -----	1,5-7

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter nal Application No
PCT/US 96/04305

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A-9012536	01-11-90	EP-A- 0466828	22-01-92
		US-A- 5421337	06-06-95
WO-A-9006718	28-06-90	AT-T- 133545	15-02-96
		DE-D- 68925586	14-03-96
		EP-A- 0449883	09-10-91
		US-A- 5419323	30-05-95
US-A-5348003	20-09-94	NONE	
WO-A-9426168	24-11-94	US-A- 5421339	06-06-95
		AU-B- 6946894	12-12-94
		CA-A- 2162922	24-11-94
		EP-A- 0702526	27-03-96